Comprehensive Analysis of Modified Hyaluronic Acid by Multidimensional Chromatography

by
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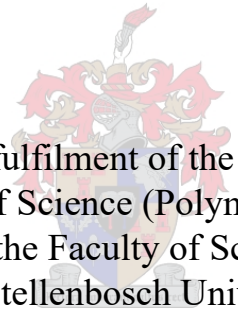

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Zanelle Viktor

December 2017

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Dedicated to my mom, without whom all this would not have been possible.

Thank you, mom, for always making the impossible, possible. Thank you, mom, for your guidance, encouragement and love.

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Abstract

Hyaluronic acid (HA) that had been modified through the hydroxyl moieties with an acrylate functional group can produce very complex and heterogeneous polymers. The substitution of the hydroxyl moieties on the HA backbone are not homogeneous, resulting in different numbers of acrylate functionalities that are present among and along the polymer chains (1^{st} and 2^{nd} order heterogeneity). Effectively, the samples have a chemical composition distribution (CCD) along with a molar mass distribution (MMD), inherited from the parent HA.

The chemical composition and molar mass distributions of a given polymer have a close correlation to the resulting final properties of the polymer. It is therefore essential to gain knowledge about the heterogeneity with respect to CCD and MMD since these distributions can provide an insight into the structure-property relationship. The properties of the polymer subsequently determine its applications.

The acrylate-functionalized HA cannot simply be characterized by a single one-dimensional LC method, for instance, gradient high performance liquid chromatography (HPLC) or size exclusion chromatography (SEC), due to the inherent complex nature of HA. It requires advanced liquid chromatographic (LC) techniques such as online two - dimensional LC (2D – LC), as it thus allows for the simultaneous determination of the degree of substitution (DS) and the molar mass distribution of the given HA sample. 2D – LC is thus a direct method to determine the CCD - MMD relationship that is present in the sample. With gradient reversed phased LC (RP – LC), as the first dimension, the modified HA was separated according to CCD i.e. DS. The resultant fractions collected from the first dimension were subjected to the second dimension, SEC, where the fractions of narrowly distributed DS were separated according to hydrodynamic volume. With the developed 2D – LC method, different structural compositions could be made apparent alongside the molar mass distribution. The developed method illustrates the heterogeneity of the modified HA samples, as bimodal molar masses were observed for narrow DS fractions.

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Uittreksel

Die substitusie van hidroksielgroepe van hialuroniese suur (HA) met 'n aktiewe akriel groep, kan komplekse en heterogeniese polimere produseer. Die substitusie van hidroksielgroepe op die HA-ruggraat is nie homogeneen nie, wat beteken dat die hoeveelheid akriel funksionaliteit wat tussen en op die polimeerkettings (1ste en 2de orde heterogeniteit) teenwoordig is, kan wissel. Gevolglik, het die monsters beide 'n chemiese samestelling distribusie (CCD) en ‘n molêre massadistribusie (MMD).

Die chemiese samestelling en molêre massadistribusie van 'n gegewe polimeer het 'n noue verband met die finale eienskappe van die polimeer. Dit is dus noodsaaklik om kennis te verkry oor die heterogeniteit van die polimeer ten opsigte van CCD en MMD. Hierdie verdelings gee insig omtrent die struktuur-eienskap verhouding, aangesien die eienskappe van die polimeer sy toepassing bepaal.

Die akriel gefunksionaliseerde HA kan nie eenvoudig gekaraktariseer word deur 'n enkele een-dimensionele vloeistof chromatografiese (LC) metode nie. Dit vereis gevorderde analitiese LC tegnieke soos aanlyn twee-dimensionele LC (2D - LC) waar gradient hoë verrigting vloeistof chromatografie (HPLC) gekoppel word aan grootte uitsluiting chromatografie (SEC). Met 2D-LC kan die graad van substitusie (DS) en die molêre massa verdeling van die HA polimeer, gelyktydige bepaal word. Dit is dus 'n direkte metode om die CCD - MMD-verhouding wat binne die monster voorkom, te bepaal. Gradiënt HPLC is gebruik vir die eerste dimensie om die HA polimeer volgens die mate van substitusie te skei. Die verskeie fraksies wat vanuit die eerste dimensie versamel is, was onderworpe aan die tweede dimensie, SEC, waar die fraksies van bepaalde DS volgens hidrodinamiese volume geskei was. Met die ontwikkelde metode kan verskillende struktuursamestellings geïdentifiseer word saam met die molêre massadistribusie daarvan. Die ontwikkelde metode illustreer die heterogeniteit van die gemodifiseerde HA monsters, aangesien bimodale molêre massa vir gegewe DS fraksies waargeneem was.

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List of Abbreviations

ACN	Acetonitrile
CC	Chemical Composition
CCD	Chemical Composition Distribution
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DS	Degree of Substitution
ELSD	Evaporative Light Scattering Detector
FT-IR	Fourier Transform Infrared Spectroscopy
GAG	Glucosaminoglycan
GlcA	β-(1-4)-D-glucuronic acid
GlcNAc	β-(1-3)-N-acetyl-D-glucosamine
HA	Hyaluronic Acid
HAM	Modified Hyaluronic Acid
HPLC	High Performance Liquid Chromatography
I.D.	Internal Diameter
LAC	Liquid Adsorption Chromatography
LC	Liquid Chromatography
LCCC	Liquid Chromatography at Critical Conditions
LS	Light Scattering
M	Molar
MALLS	Multi-angle Laser Light Scattering
MeOH	Methanol
MM	Molar Mass
MMD	Molar Mass Distribution
NMR	Nuclear Magnetic Resonance Spectroscopy
NP	Normal Phase
RC	Regenerated Cellulose
RI	Refractive Index Detector
RP	Reversed Phase
rpm	Revolutions per Minute
SEC	Size Exclusion Chromatography
THF	Tetrahydrofuran
2D-LC	Two - Dimensional Liquid Chromatography

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List of Symbols

Å	Angstrom
c	Concentration
C_M	Concentration of Analyte in Mobile Phase
C_S	Concentration of Analyte in Stationary Phase
Đ	Dispersity Index
ΔG	Change in Gibbs free energy
ΔH	Change in Enthalpic Interactions
K_d	Distribution Coefficient
K_{LAC}	Distribution Coefficient as a Function of Enthalpic Interactions
K_{SEC}	Distribution Coefficient as a Function of Entropic Interactions
M_i	Molar Mass of Given Chain Length
M_n	Number-Average Molar Mass
M_w	Weight-Average Molar Mass
N_i	Number of Molecules
R	Universal Gas Constant
ΔS	Change in Entropic Interactions
T	Absolute Temperature
V_R	Retention Time
V_e	Retention Volume/Elution Volume
V_i	Interstitial Column Volume
V_p	Pore Volume of Packing

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Chapter 1

Introduction and Objectives

In this chapter, a general introduction to hyaluronic acid and the characterization thereof are briefly discussed. The outline of the objectives of the study and summary of the thesis layout are also presented.

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1.1. Introduction

Polysaccharides are a form of polymeric materials that occur in nature. Hyaluronic acid (HA), specifically, is part of the glycosaminoglycan family of polysaccharides which are linear and unbranched polymers consisting of disaccharide repeat units.[1-4] HA is used extensively in the medical and cosmetic industries due to its physiochemical and viscoelastic properties. However, being a natural polymer it lacks certain physical and mechanical properties, which limits its applications. To overcome these shortfalls, HA is typically modified at the carboxylic acid or hydroxyl moieties to enhance its properties for a given application.[5,6] Although the modification of HA is well understood, the characterization of such modified natural polymers still remains a challenge as these complex HA are not only heterogeneous with regards to both molar mass and chemical composition, but also the solubility of these polymers in hydrophobic or hydrophilic media change with modification.[5]

Characterization of hyaluronic acid aims to correlate certain molecular properties of the polymer, such as molar mass distribution, chemical composition, and the degree of substitution or modification, to the structure of the polymer. These fundamental characteristics of the polymer play a critical role in the final properties of the material and as such knowing these characteristics and corresponding structure-property relationships allows for a better understanding of the final material's behaviour.[7,8] In order to accomplish a comprehensive characterization of hyaluronic acid, advanced chromatographic techniques need to be utilized.

Characterization of hyaluronic acid and its derivatives has previously focused on utilizing techniques such as liquid chromatography (LC),[9] Fourier transform infrared spectroscopy (FT-IR)[10] and nuclear magnetic resonance spectroscopy (NMR),[11] all of which only characterize the polymer according to one single property. By developing an online 2D-LC approach, comprehensive information on modified and unmodified HA in terms of molar mass and chemical composition can be obtained. A 2D-LC approach would use liquid chromatography (HPLC) as the first dimension and size exclusion chromatography (SEC) as the second dimension in order to determine composition and molar mass distributions, respectively.

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Therefore the aim of the study was to develop a suitable and robust multi-dimensional LC protocol for the comprehensive analysis and separation of HA and HA modified with acrylic moieties to obtain comprehensive information of all chemically distinct fractions regarding molar mass and degree of substitution. Multi-dimensional LC has been successfully applied to synthetic polymers and natural polymers such as cellulose. To our knowledge, no study has been reported yet in which multi-dimensional LC has been employed to achieve separation and characterization of HA functionalized with acrylate moieties. Thus, the combination of the LC characterization techniques will provide the necessary comprehensive information regarding the heterogeneity of the complex HA polymers. The knowledge gained from the multi-dimensional analysis could assist in understanding the structure-property relationships, which are much needed to determine the end use and application of the HA polymers.

1.2. Objectives

The project entailed the following:

(1) Perform solubility studies on the unmodified HA and the modified HA samples to determine the degree of dissolution of the samples in a given solvent system by dynamic light scattering (DLS).

Here the aim is to find a suitable solvent system that would be able to dissolve both the unmodified HA and the modified HA with different degrees of substitution. In addition, the solvent system must be suitable for 2D-LC analysis, in other words, it will be used for both chemical composition characterization in the first dimension (HPLC) and molar mass characterization in the second dimension (SEC).

(2) Develop a SEC method that would be compatible with an ELSD detector and the 2D-LC setup. Search for a volatile salt which is ELSD compatible and which optimizes separation.

(3) Develop a HPLC method which separates according to hydrophobicity using a non-polar reversed stationary phase.

(4) Perform online coupling of HPLC and SEC to obtain comprehensive information of all chemically distinct fractions regarding molar mass and degree of substitution.

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1.3. Thesis Layout

This thesis consists of the following seven chapters:

Chapter 1

A general introduction to the study conducted, followed by the objectives and explanation of the layout of the thesis.

Chapter 2

A detailed background of hyaluronic acid related to its structure, modification and application is presented. Included in the review is a brief theoretical background on the principles of the analytical techniques used for the characterization of the HA polymers.

Chapter 3

The solubility studies conducted on the HA polymers are discussed with the main focus on finding volatile salts and the two different methods, visual examination and dynamic light scattering (DLS), used to establish the most promising solvent system, are compared.

Chapter 4

The SEC method development for the molar mass determination of the HA polymers is presented and discussed. With the aid of SEC coupled with both dRI and ELSD, the effect of salt on the characterization of the HA polymers is investigated, as the salt assisted in reducing aggregate formation, which is inherent to HA.

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Chapter 5

The different steps followed in the development of a gradient HPLC method on a non-polar column to separate the unmodified HA from the modified HA as well as the characterization of the HA polymers according to chemical composition are presented and discussed.

Chapter 6

The optimization of the reversed phase HPLC method developed for the two-dimensional liquid chromatography (2D-LC) analysis is shown and discussed. Also included are the optimization of the 2D-LC methods and the discussion of the 2D-LC results obtained.

Chapter 7

The results in Chapters 3 through 6 are briefly summarized and concluded followed by proposed future work related to the study.

1.4. References

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Chapter 2

Historical and Theoretical Background

The historical and theoretical background is based on hyaluronic acid and acrylate-modified hyaluronic acid. A brief introduction to polysaccharides mainly focussing on hyaluronic acid (HA) will be given. The introduction will concentrate on the structure, modification, functionality and application of HA as well as the characterization thereof. A theoretical background with regards to the liquid chromatography techniques used to characterize HA macromolecules is provided.

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2.1. Introduction to polysaccharides

Polysaccharides are biomacromolecular carbohydrates that consist either of monosaccharide repeat units, homopolysaccharides, or disaccharide repeat units, heteropolysaccharides.[1] The repeating units of polysaccharides can be bound by different types of glycosidic bonds (alpha (α) or beta (β)) and can be linked by different types of linkages (1→2, 1→3 or 1→4) to form the macromolecular chain.[1,2] Polysaccharides are thus very complex, heterogeneous macromolecules as their molecular structure can range from linear to highly branched macromolecules,[1,3] and thus differ in chemical composition and molar mass distribution.[1,4]

Polysaccharides can be obtained from numerous natural sources including organisms such as plants, and bacteria.[1-3] Due to their natural occurrence, they have a broad scope of biological benefits and physiochemical properties. The biological benefits of polysaccharides include antibiotic effects, antioxidant capabilities, biodegradability, and biocompatibility.[2, 5-7] The diverse physiochemical properties of polysaccharides and the fact that they can be obtained from natural resources as well as be chemically or enzymatically modified, have gained significant interest from industry.[2,8] Hence polysaccharides have seen numerous applications in fields such as biomedicine, pharmaceutics and cosmetics.[1]

Polysaccharides are important in assisting in biological functions of organisms and are classified according to their function. The two most important functions are storage and structural.[3] Storage polysaccharides stores the energy required for biological processes e.g. starch and glycogen. Structural polysaccharides, such as cellulose, chitin and hyaluronic acid, are rigid structures which provide extracellular support and shape to cells, tissues and organs in all organisms.[1-3]

2.1.1. Polysaccharide derivatives, hyaluronic acid

A natural polysaccharide derivative, hyaluronic acid (HA), is classified as a glycosaminoglycan (GAG). [2,9] HA is a linear and unbranched heteropolysaccharide which can have an average molar mass up to 10^7 $g.mol^{-1}$.[10-12] It consists of two alternating monosaccharide units of an acidic sugar, D-glucuronic acid (GlcA), and an acetylated aminosugar, N-acetyl-D-glucosamine (GlcNAc), which are bound by a glycosidic bond,

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forming the disaccharide repeating unit.[12-14] The disaccharide repeating unit, Glucuronate - β - (1→3) - GlcNAc is linked in β-(1→4) linkages,[3,13,15,16] due to the stereochemistry of the acetyl group, which results in the extended left-handed helix macromolecular structure of HA (see **Figure 2.1**).[18] The helix is stabilized by the hydrogen bonding network that forms in an aqueous medium.[15] The hydrogen bonding arises from the attraction between the hydrogen of water and the more electronegative atoms present in the alcohol (–OH), acetamido (–CO–NH–) and carboxyl (–COOH) groups. In aqueous solution, HA appears as an expanded random coil.[2,15,19-21]

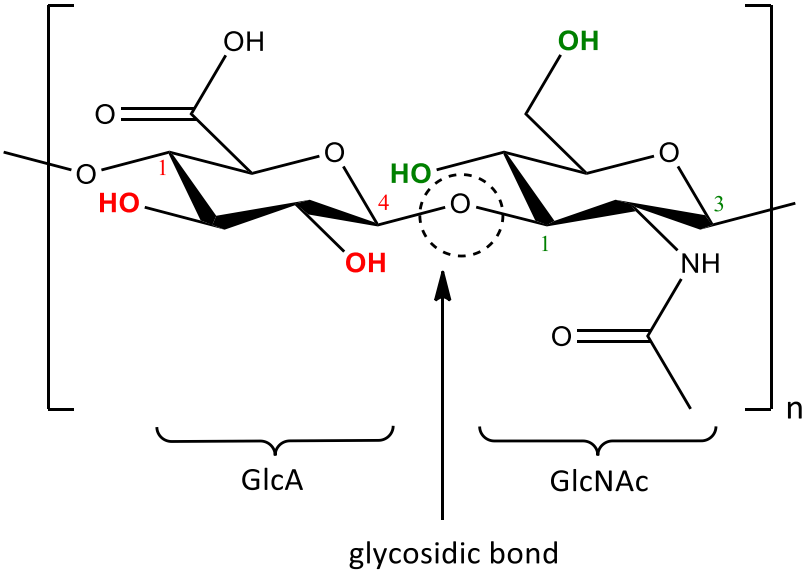

Figure 2.1 *Disaccharide repeating unit of hyaluronic acid which consist of β -(1→4)-D-glucuronic acid (GlcA) and β -(1→3)-N-acetyl-D-glucosamine (GlcNAc), bonded by a,β- glycoside bond.*

After the discovery of HA in 1934 by Meyer and Palmer,[10,19,22,23] HA has been found to be a component of not only the vitreous humour, but also of connective tissues, joints, and skin.[2,17,19] Since HA occurs naturally in the human body, it assists in a wide range of biological functions, which include acting as structural support for connective tissue, lubricant for joints, shock absorber and it provides strength and elasticity to cartilages.[12,15,19,24] It is thus evident from the biological versatility of HA in the human body, that it has unique biochemical and physical properties.[2] The viscoelastic, hydroscopic and mucoadhesion properties are unique to high molar mass HA (10,000 $g.mol^{-1}$ and upwards).[10,13,25-27] Subsequently, high molar mass HA has found use in numerous

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applications in the medical, pharmaceutical, and cosmetic fields.[10,12] HA has been used as a chemical substance for the treatment of wounds, burns and osteoarthritis.[19,24] As previously stated, HA is hydroscopic, which means it has the ability to retain moisture.[2,15,28] Thus, making it attractive for cosmetic application as HA hydrates the skin and functions as a cushioning agent as well as nourishes the skin without being toxic or immunogenic.[15,29] Low molar mass HA (2000-3500 $g.mol^{-1}$, oligosaccharide) is generally used in inflammatory medicine and is known to assist in angiogenesis.[10,25,27]

However, even though HA is readily available in natural resources, and was generally extracted from rooster comb, the extraction thereof was difficult, expensive and environmentally unfriendly.[10,19,30] Alternative methods for generating HA, included microbial fermentation and the extraction of HA from bacteria such as *Streptococcus.*[10,30,31] In addition, HA lacks certain mechanical and physical properties required for specific applications. Thus, to overcome these shortcomings, the parent HA is often modified, resulting in HA derivatives.

2.1.2. Derivatives of hyaluronic acid

The potential of utilizing HA for biological applications has led to research to the modification of HA to produce HA derivatives which improve the properties while maintaining the biocompatibility, viscoelasticity and biological activity of HA.[16,17,19,24] In 1950, Jeanloz and Forchielli, were one of the first to report on the modification of HA. They modified HA via the carboxylic groups to obtain methylated, acetylated and triphenylchloromethylated HA derivatives.[32,33]

HA can be chemically modified via crosslinking or conjugation.[13] During conjugation the modifying group is grafted onto the macromolecule backbone by a single bond, while during crosslinking, the grafting process results in the macromolecule chains being bonded together by multiple bonds via the crosslinking agent.[13] HA can be modified through three possible target sites, the carboxylic acid on the D-glucuronic acid monosaccharide unit, the primary and secondary hydroxyl moiety and the N-acetyl group on the N-acetyl-glucosamine monosaccharide unit.[1,13,16,34] The modification through the D-glucuronic carboxylic acid functionality is mainly achieved by amidation and esterification.[16,17] Amidation and

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esterification occurs by carbodiimide- and allylation-mediated chemistry, respectively.[16,35] The primary and secondary hydroxyl moieties are generally modified by etherification using bisepoxides, resulting in crosslinking, or esterification using methacrylic anhydride. Alternatively, the hydroxyl groups are modified by divinylsulfone crosslinking agents.[13,16,17,35] The primary alcohols are more accessible than the secondary alcohols which are present on the N-acetyl-glucosamine monosaccharide, and thus have a faster rate of substitution.[13] The N-acetyl group on the N-acetyl-glucosamine monosaccharide can undergo a deacetylation/amidation reaction. The deacetylation of the N-acetyl group is performed using hydrazine sulphate, after which it can be reacted with an acid, similar as in the amidation reaction.[1,13,34]

The structural modifications which enhance the physiochemical properties and the mechanical properties are dependent on the following structural parameters: (1) nature of the modifying compound, (2) degree of substitution (DS), (3) chemical heterogeneity and (4) the overall molar mass of the macromolecules. The nature of the compound with which the HA is modified can influence the hydrophilicity of HA, e.g. benzylic acid used during esterification increases the hydrophobicity, reducing the water solubility of HA.[13,16,36,31]

Another example is crosslinking HA using divinylsulfone, which increases the stability of HA and its mechanical properties. The stability of HA is usually 1 to 2 days in soft tissue,[17] and by crosslinking HA, an insoluble hydrogel is obtained which prolongs HA degradation.[13,16] The structural parameter DS refers to the extent to which the chemical modification has occurred per repeat unit. HA is generally considered to be heterogeneous with regards to molar mass, thus polymer of various chain length can be found in one single sample of HA. The chemical modification can occur among the different HA chains present in a given sample (1st order heterogeneity), as well as along the HA backbone (2nd order heterogeneity) to various degrees, thus resulting in a diverse range of chemical compositions and molar masses. For the present study, the –OH moiety was modified with an acrylate functional group to various degrees. Thus the average DS per repeating unit for the given HA derivative can be expressed as follow in equation 2.1:

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$$DS = \frac{(number\ of\ substituted\ groups)}{(number\ of\ HA\ repeat\ units)} \quad 2.1$$

It is evident that HA derivatives attained from the same parent HA can be heterogeneous, as they consist of a mixture of different chemical compositions, chain lengths and DS. Thus the differences in the heterogeneity of the macromolecules result in unique characteristic properties which determine their performance and subsequently the suitable application they can be used for. By understanding the structure-property relationships, advances can be made in the production and industrial application of HA derivatives. In order to understand this relationship, comprehensive characterization is required. For the characterization of the HA derivatives the heterogeneity with regards to DS, CCD and MMD can be determined, in addition to establishing how each distribution correlates to one another.

2.1.3. Characterization of hyaluronic acid derivatives

HA derivatives are complex macromolecules and are heterogeneous with regards to several structural characteristics, such as DS, molar mass and chemical composition. HA derivatives can be characterized using spectroscopy and chromatographic techniques. As previously stated, the study focuses on the characterization of HA functionalized with an acrylate moiety.

HA has been previously characterized,[13,22,27,37,38] however the characterization reports of HA derivatives by liquid chromatography for both chemical composition and molar mass are limited. The average DS for modified HA has been determined by using nuclear magnetic resonance spectroscopy (NMR),[39-42] Fourier transform infrared spectroscopy (FTIR)[39,42] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[43] These methods are based on the detection of the functional substituted groups or the element present in the HA derivative. Bencherif et al.[44] and Fenn et al.[45] used ^{1}H NMR spectroscopy to determine the degree to which the HA was modified with the methacrylate group. Zwako et al.[43] used both ^{1}H NMR and MALDI-MS to determine the degree to which the HA was modified with the derivatized cyclodextrin. Oudshoorn et al.[41] determined the average DS of methacrylate-functionalized HA with reversed phase high performance liquid

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chromatography (RP-HPLC). However, to determine the DS, the methacrylate-functionalized HA was hydrolyzed. Finelli et al.[46] determined the DS of hexadecylamide HA derivative with HPLC/fluorimetry. Prior to analysis, the hexadecylamide HA derivative was hydrolyzed.

For the determination of the molar mass and molar mass distribution of derivatized HA, size exclusion chromatography (SEC) was hyphenated to a multiangle laser light scattering (MALLS) detector.[38,40,47] The reason for using MALLS as a detector is that it provides the absolute molar mass when calculated with the relevant refractive increment values (dn/dc).[13] Šedová et al.[48] determined the molar mass of hyaluronan (HA) polyaldehyde and the distribution thereof by SEC-MALLS. The mobile phase comprised of aqueous 50 mM sodium phosphate with 0.02 % sodium azide.

2.2. Basic separation principles for liquid chromatography

Liquid chromatography uses a stationary phase and a mobile phase. A sample, comprising of a mixture of components, is introduced onto a column (stationary phase) via the mobile phase. The mobile phase continuously flows through the column, and forces the different components, present in the sample, to migrate through the column. Based on the interaction of the individual components in the sample, with the stationary phase, the sample is separated into its respective constituents. Hence, separation is governed by the distribution of the analytes between the mobile phase and the stationary phase.[49] An analyte within a sample has a different affinity towards the mobile and stationary phases, and as a result, has a unique equilibrium constant. Subsequently, the analytes have different retention volumes and the mixture is separated into its constituents. The distribution and affinity of the analytes for a given stationary phase is expressed by the distribution coefficient, K_d.[50,51]

$$Kd = \frac{C_S}{C_M} \qquad 2.2$$

where C_S and C_M refer to the concentration of the analyte in the stationary phase and mobile phase, respectively.

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Analytes which have a stronger affinity towards the stationary phase than the mobile phase will be retained longer than the analytes which have more affinity towards the mobile phase and will elute later. The affinity of the analyte for the mobile phase is related to solvent quality. A mobile phase can either consist of a single solvent or a combination of thermodynamically "good" and "poor" solvents. If the mobile phase is thermodynamically "good" for the analyte, the affinity of the analyte for the mobile phase will be larger than its affinity for the stationary phase. If the mobile phase is thermodynamically "poor" for the analyte the interaction forces of the analyte are more towards the stationary phase. The retention volume of the solute can be described by the following equation 2.3.[50-52]

$$V_R = V_i + V_p K_d \qquad 2.3$$

where V_R is the retention volume of the solute, V_i is the interstitial volume of the column, V_p is the pore volume of the stationary phase and K_d is the distribution coefficient. The distribution coefficient relates to the change in Gibbs free energy which expresses the retention behaviour of the analytes in terms of change in entropy (ΔS) and change in enthalpy (ΔH). The enthalpy change relates to the interaction (via partitioning or absorption) of the analytes with the active sites of the stationary phase, while the entropy change relates to the possible conformations which the analytes can occupy. However, not all conformations are possible due to the restraints caused by the pore sizes of the stationary phase. Thus the separation of the analytes can be either due to enthalpic interactions or the conformational entropy or a combination of both. The change in Gibbs free energy is expressed as follows:[52,53]

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_d \qquad 2.4$$

$$\ln K_d = -\frac{\Delta G}{RT} = \left(\frac{\Delta S}{R} - \frac{\Delta H}{RT}\right) \qquad 2.5$$

Where ΔH is the change in enthalpy, ΔS is the change in entropy, R is the gas constant and T is the absolute temperature of the system.

Based on the various possible interactions of the analyte molecules with the stationary and mobile phase, different modes of separation can be attained. The mode of separation is

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determined by the degree to which the enthalpic and entropic effects contribute to the change in Gibbs free energy. Thus the general expression for the distribution coefficient (K_d) is as follows:[53]

$$K_d = K_{SEC} \cdot K_{LAC} \quad 2.6$$

where K_{SEC} is associated with the entropic effects, while K_{LAC} is associated with the enthalpic effects.

The different modes of separation include, size exclusion chromatography (SEC), liquid chromatography at critical condition (LCCC) and liquid adsorption chromatography (LAC) and is illustrated in **Figure 2.2.**[54-56]

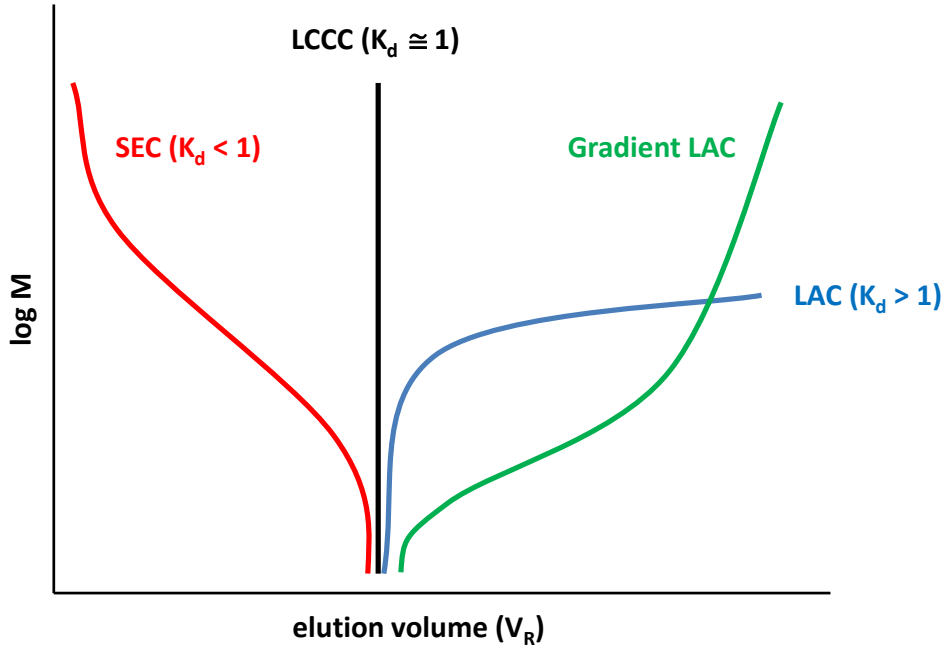

Figure 2.2 *Illustration of the different modes of separation for liquid chromatography i.e. SEC, LCCC, LAC and gradient LAC.*[51,53]

2.2.1. Size exclusion chromatography (SEC)

SEC is a LC technique that is used to determine the molar mass distribution of a given polymer sample. The separation in SEC is governed by the hydrodynamic volume of the molecule in solution, in other words the conformational entropy (conformational changes).[56] A macromolecule which is dissolved in a thermodynamically "good" solvent will

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have negligible contributions from enthalpic effects (stationary phase – polymer interaction) and ΔH becomes insignificant, which effectively means the molecule is separated based on its conformational changes. Molecules with larger hydrodynamic volumes are excluded as they cannot penetrate the porous stationary phase (K_{sec} = 0), and elute first from the column. Small molecules with much lower hydrodynamic volumes are able to penetrate the porous stationary phase completely (K_{sec} = 1) and elute last from the column. The separation range of SEC is thus 0 < K_{sec} < 1 and is illustrated in **Figure 2.3**.

The retention volume for SEC is described by the following equation:[52,53]

$$V_R = V_i + V_P K_{SEC} \quad 2.7$$

$$K_{SEC} = exp\left(\frac{\Delta S}{R}\right) \quad 2.8$$

where V_R is the retention volume of the solute, V_i is the interstitial volume of the column, V_p is the pore volume of stationary phase and K_{SEC} is the distribution coefficient. ΔS is the change in entropy and R is the gas constant.

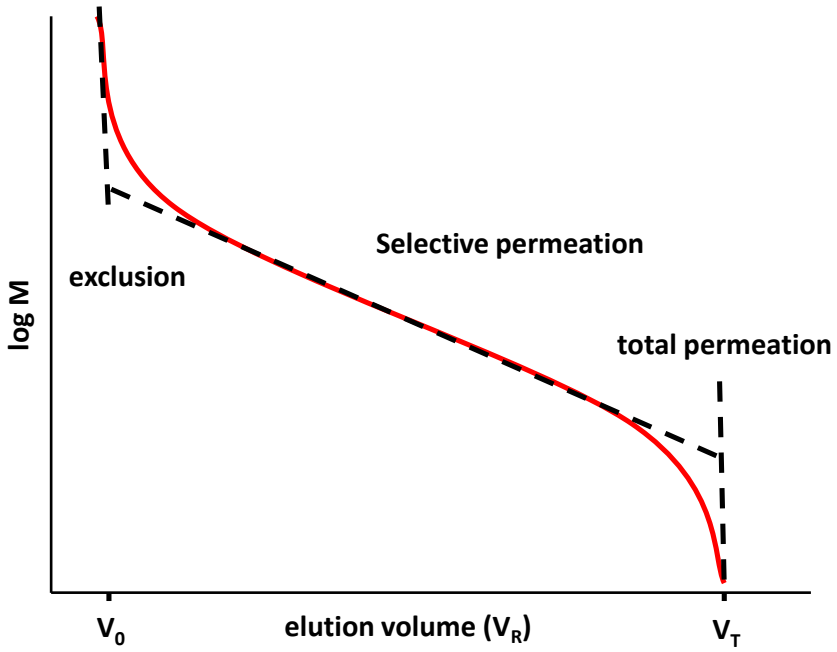

Figure 2.3 *A representation of a typical SEC calibration curve illustrating the separation range of SEC, 0 < K_{sec} < 1.*[57]

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Thus, with SEC detailed information about the molar mass distributions such as number-average molar mass (M_n), weight-average molar mass (M_w) and the dispersity (Đ) are obtained. To determine the relative molar mass of a given polymer sample, calibration with narrowly distributed polymeric standards of known molar masses is required. SEC is therefore described as a relative method as the molar mass determined, is relative to that of the calibration standards. M_n, M_w and Đ are described by the following equations:[52,54,58,59]

$$\overline{M_n} = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad 2.9$$

$$\overline{M_w} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} \quad 2.10$$

where N_i is the number of molecules with a given molar mass, M_i, of a given chain length. The dispersity of a given polymer is determined by the ratio of M_n to M_w with dispersity equal to 1 representing a monodisperse sample. The dispersity is expressed by the following equation:[52,54]

$$Đ = \frac{M_w}{M_n} \quad 2.11$$

SEC has several limitations when it comes to the analysis of complex macromolecules. The molar mass determined by SEC is not an absolute representation of the molar mass of the macromolecules, as the chemical composition of the macromolecule is typically different to that of the calibration standards. Thus the apparent molar mass determined is inherently inaccurate. In addition, SEC does not have the ability to separate according to chemical composition, architecture and functionality, subsequently, macromolecules of different chemical compositions, but same hydrodynamic volumes, elute at the same retention volume.[54,60]

2.2.2. Liquid adsorption chromatography (LAC)

Liquid adsorption chromatography (LAC) is defined by the separation of the macromolecules based on their interactions with the stationary phase and mobile phase. The separation in LAC is governed by the enthalpic effects or in other words the stationary phase – analyte

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interactions. Therefore, in an ideal LAC, the contribution from the conformational changes to the change in Gibbs free energy becomes negligible and as a result, ΔS is insignificant (ΔS = 0). The distribution coefficient is expressed only by the enthalpic effects:[52,53]

$$K_{LAC} = exp\,(\frac{-\Delta H}{RT}) \qquad 2.12$$

The interaction of the analytes with the active sites of the stationary phase is based on the chemical composition of the analytes and the affinity thereof with the stationary phase. Thus LAC is used to separate macromolecules with regards to chemical composition. Molecules with a greater affinity towards the stationary phase usually experience adsorption and hence are more retained, and elute last from the column. Molecules with a little affinity towards the stationary phase will elute first. Consequently, each chemically different analyte within a polymer sample will have a different retention volume. The more repeat units with functional groups there are along the macromolecular chain which has an affinity to the stationary phase; K_{LAC} will increase due to the increase in the retention factor as a result of the macromolecule being more retained. The same behaviour can be seen for macromolecules of the same chemical composition and hence selectivity, but different molar masses. The higher molar mass molecules will have more interaction sites to interact with the stationary phase and will be more retained than the macromolecules of the same composition but of lower molar mass.[61-63] The interaction of the high molar mass macromolecules can be very strong and requires the modification of the mobile phase strength to desorb the analyte and elute it from the column. Therefore, isocratic LAC is suitable for the separation of small, low molar mass macromolecules, while gradient LAC is required for larger, high molar mass macromolecules. A gradient can be achieved by either altering various parameters of the mobile phase, which include the composition, pH and ionic strength, or by changing the operating temperature. The separation in gradient LAC is governed by gradually changing the equilibrium between the macromolecule-stationary phase interaction and the macromolecule-solvent interaction. The adsorption of the macromolecules to the stationary phase is strong in a weak initial mobile phase composition and with an increase in mobile phase strength the macromolecules start to desorb if sufficient eluent strength is reached, and separate elution is achieved.[56,64]

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The concept of solvent gradient LAC was first introduced by Glöckner[55] and has successfully been utilized for the characterization of complex, heterogeneous synthetic as well as natural macromolecules. The separation of natural polymers such as modified cellulose has been conducted by gradient LAC to separate the unmodified from the modified cellulose as well as to determine the degree of substitution (DS). Ghareeb et al.[65] and Shakun et al.[66] both used gradient LAC to determine the DS of derivative celluloses.

2.2.3. Multidimensional liquid chromatographic techniques

Complex macromolecules have multiple distributions, which are molar mass distribution, chemical composition distribution, functionality type distribution or architecture distribution. The various distributions are all interconnected. The hyphenation of chromatographic techniques allows for efficient and comprehensive characterization of these complex macromolecules.[53,67] By coupling two chromatographic techniques, the different distributions can independently be characterized, while providing information on how one distribution, for example, chemical composition, correlates to another distribution, molar mass. With the aid of two-dimensional liquid chromatography (2D-LC), separation according to chemical composition and molecular size distribution can be simultaneously obtained. [53,67]

A general 2D-LC configuration for the analysis of macromolecules is with gradient HPLC as the first dimension and isocratic SEC as the second dimension.[55] The two dimensions are coupled via a transfer valve (see **Figure 2.4**). During 2D-LC a macromolecule is first separated according to one structural parameter, chemical composition, in the first dimension. Fractions collected from the first dimension can either be transferred or stored by an electronically controlled transfer valve consisting of two storage loops. Thus a fraction collected previously in the one loop is transferred to the second dimension and separated according to a different structural property, e.g. hydrodynamic volume.[51,53,68] While the fraction in the first loop is analysed by the second dimension, the second loop is being filled. It is therefore important that the analysis time of the second dimension should be equal or faster than it takes to collect a fraction from the first dimension. [51,53,68] By calibrating the second dimension, the molar mass distribution of the macromolecules can be attained.

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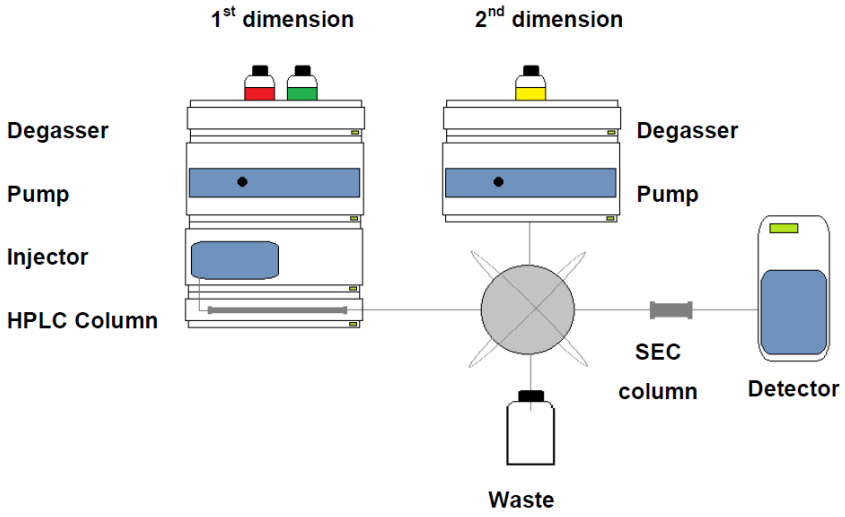

Figure 2.4 *Illustration of a typical online multi-dimensional liquid chromatography configuration (2D-LC). The 1st dimension generally separates according to chemical composition and the 2nd dimension according to hydrodynamic volume.*[69]

The application of multi-dimensional liquid chromatography for the comprehensive characterization of natural polymers has been reported.[67] Greiderer et al.[70] used 2D-LC to investigate the temperature dependence of hydroxypropylmethylcellulose (HPMC), a polysaccharide. First dimension separation was achieved using a reversed phase Zorbax-RX C-8 column with a linear gradient elution profile consisting of 0.05 % trifluoroacetate (TFA) in water and 1-propanol. Ghareeb et al.[71] performed 2D-LC of cellulose acetate, a polysaccharide, being separated according to DS (DS range = 1.53 – 2.92) in the first dimension with gradient HPLC followed by the separation of the individual fractions according to size in the second dimension. Separation in the first dimension was achieved on a pure silica stationary phase, with a multi-step eluent gradient, initially comprising of dichloromethane (DCM) to methanol (MeOH). For the second dimension, a PSS-GRAMS column with an isocratic mobile phase of 45 % dimethyl sulphoxide (DMSO) and 55 % 1,4-dioxane with 50 mmol/L ammonium acetate was used to separate the fractions according to size. Characterization of sodium carboxymethyl cellulose by comprehensive 2D-LC has been performed by Shakun et al.[72], where the study focused on investigating the effect of molar

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mass on the elution behaviour. A Luna PFP column was used to separate the polymer according to DS in the first dimension and a PSS Suprema column was used in the second dimension to separate the fractions according to hydrodynamic volume. The gradient eluent composition for the first dimension was H_2O:MeOH, 69.5:30.5 (vol. %) with 100 mmol/L of both ammonium acetate and acetic acid. An isocratic eluent composition of 100 mmol/L aqueous ammonium acetate was used for the second dimension. No studies could be found on the characterization of acrylate HA derivatives by multi-dimensional liquid chromatography.

2.2.4. Detectors used for liquid chromatography analysis of polymers.

Detection of the solute after being subjected to LC is as important as the LC method chosen for the analysis such as SEC or LAC. The choice of an appropriate detection method may allow for additional insights into the nature of the solute such as concentration, molar mass or even chemical composition.[52,53,73,74]

Concentration sensitive detectors are extremely useful in LC as the vast majority of these detectors measure a certain spectroscopic property of the solute to facilitate detection. These spectroscopic properties are directly tied to the nature of the solute such as specific functionalities present. Ultraviolet (UV) and infrared (IR) detectors operate on this principle and measure the transmittance/absorbance at a chosen wavelength that will interact with a given functional group on the solute.[73] Since the Beer-Lambert law correlates the concentration to the attenuation of the light, IR and UV detectors are also capable of measuring the concentration of a solute in LC,[73] making them effective and versatile. However, UV and IR detectors lack universality in the sense that for the solute to be detected, known functional groups must exist on the solute that is either IR or UV active, narrowing their scope of use.

There exist several concentration dependent universal detectors for LC with two of the most common detectors being the differential refractive index detector (dRI) and evaporative light scattering detector (ELSD).[73] They are classified as universal since their means of detection are independent of the type of solute being detected.

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The dRI detector operates solely on the principal that a given solute will alter the refractive index of the solution based on the concentration of the solute and its own refractive index properties.[73] The detector operates as follows. The refractive index of both flow cells are measured with a light source, typically a laser and the difference between the detector cell and the reference cell is recorded. If no solute is present in the eluent passing through the detector cell then the detector cell and reference cell will have the same refractive index and no solute is detected. However, when there is a solute present in the eluent, the refractive index of the eluent changes proportionally to the concentration of the solute and the nature of the solute's refractive index, allowing for a difference between the reference cell and the detector cell to be observed. RI detectors are among the most useful detectors available to LC. However two major drawbacks to RI as detector is its lack of sensitivity, as the change in refractive index with a minute amount of solute present must be observable, and that it is not compatible with gradient LC.

To overcome the sensitivity drawback of RI detectors, evaporative light scattering detectors provide an alternative means to detect minute amounts of analyte.[73] ELSD operates on the principle that a particle of a given size will scatter light away from the parent beam when passing through it. The ELSD operates generally as follows. The eluent containing the solute passes from the column into a nebulizing chamber where the eluent containing the solute is finely dispersed into droplets. These droplets are carried along a heated drift tube which is set above the boiling point of the mobile phase. In the heated drift tube, the volatile mobile phase is removed while the non-volatile solute remains as particles. These solute particles then pass through a laser and the scattered light from the particles is detected.[75-78]

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Chapter 3

Solubility Studies and Dynamic Light Scattering Analysis

This chapter will discuss solubility studies conducted on various HA polymeric samples. The discussion will mainly focus on the effect of the addition of an electrolyte to the solvent systems investigated and the methods used for the determination of the most suitable solvent system for the dissolution of HA polymeric samples. The methods used include visual examinations and dynamic light scattering (DLS experiments).

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3.1. Introduction

The main objective of this study was to develop an online multidimensional liquid chromatographic technique, coupled to an ELSD, for comprehensive characterization of hyaluronic acid (HA) and acrylate-functionalized HA derivatives. In order to analyse the HA polymers according to chemical composition (CC) and molar mass distribution (MMD) by liquid chromatography, the polymer sample must be completely in solution. A suitable solvent system was required that would dissolve all modified HA samples irrespective of the degree of acrylate substitution (DS = 0 – 4). Finding a suitable solvent system was a challenging task as the modification of the hydrophilic HA polymer samples with a hydrophobic acrylate moiety significantly influences the solvation properties of the HA polymers.[1,2,3] The hydrogen bonding network formed by HA and modified HA samples[4] impeded the determination and selection of a suitable solvent system for these particular species of polymer samples. The following criteria were imposed on a suitable solvent system; (1) complete dissolution of the polymeric samples, (2) suppression of secondary interactions such as hydrogen bonding, (3) minimization of polymer degradation and, most importantly, (4) compatibility with the stationary phase and ELSD detection system.

An extensive solubility study on the HA polymers has been conducted previously by members of the Pasch group.[5] Based on the results obtained from the solubility studies, it was concluded that the most suitable solvent system for the dissolution of the HA polymers was DMSO:H_2O (60:40, v/v %). In the molar mass determination study it was found that with the addition of a salt to the sample solvent, the formation of aggregates was suppressed, therefore, a true representation of the molar mass range of HA and modified HA polymers could be ascertained.[5] It was concluded that DMSO:H_2O (60:40, vol %) with 0.05 M lithium bromide (LiBr) was the most suitable solvent system for the complete dissolution of all HA polymeric samples.[5] The addition of an electrolyte assisted in disruption of the extensive hydrogen bonding networks present in HA polymers.[5,6] The developed solvent system, however, was not compatible with the ELSD detector system, as LiBr is a non-volatile salt and could not be evaporated by the ELSD. This meant that the non-volatile LiBr could interfere with the sample signal detected by the ELSD. Besides the possible interference from LiBr, DMSO was chosen as the sample solvent and mobile phase solvent could not completely be evaporated by the available ELSD (maximum operating temperature of 100

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°C) due to the high boiling point of 189 °C of DMSO. For this reason, the solubility studies on the HA polymers were continued in search of a suitable solvent system for both dissolution and LC analysis, that would be compatible not only with the ELSD and stationary phase but could also be used as a mobile phase for both the first dimension and second dimension in multi-dimensional LC.

3.2. Experimental and Instrumentation

3.2.1. Solvents and Chemicals

Acetonitrile (ACN) (HPLC grade, Sigma-Aldrich), 1,4-dioxane (HPLC grade, Sigma-Aldrich), water (H_2O) (Millipore from laboratory H_2O filtration system), ammonium formate (NH_4HCO_2) (Sigma-Aldrich), ammonium acetate ($NH_4CH_3CO_2$) (Sigma-Aldrich), ammonium trifluoroacetate ($NH_4CF_3CO_2$) (Sigma-Aldrich), formic acid (CH_2O_2) (Merck), trifluoroacetic acid (CF_3COOH) (Sigma-Aldich). The solvents and chemicals were used as received.

3.2.2. Dynamic Light Scattering Instrumentation

All DLS measurements were performed on a Malvern Zetasizer S173 Nano series at a temperature of 40 °C. Each sample was equilibrated for 10 minutes at 40 °C before the measurement was conducted. The size (nm) was measured for both the unfiltered samples and the filtered samples. Data acquisition was conducted on Zetasizer software (Version 7.11). Data processing was done on Origin Pro 8.0.

3.2.3. Sample Preparation

A sample concentration of 1 $mg.mL^{-1}$ was prepared by dissolving 5 mg of HA sample in 5 mL of the selected solvent system (listed in **Table 3.2**), stirred for 24 hours at 500 rpm. The samples were dissolved at both 25 °C and 40 °C, respectively. A temperature of 40 °C was chosen for the dissolution study to determine if the dissolution of HA samples improved at an elevated temperature. From previous studies carried out on the HA polymer samples, it was shown that the samples degrade above 40 °C and for this reason 40 °C was chosen as the maximum dissolution temperature.[5,7] Throughout the study, the salt concentration was

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varied depending on both the nature of the sample (pH sensitive, see **section 3.2**) and the results obtained.

3.2.4. Samples

The HA samples were provided and synthesised by L'Oréal (Paris, France). HA was chemically modified through the hydroxyl (–OH) groups (see **Figure 3.1** and **Figure 3.2**) via esterification with an acrylate functionality.[5,10] For the purpose of the solubility study, a representative selection was made from the series of unmodified and modified HA samples received as limited amounts of each sample was available. The HA samples with varying degrees of substitution can be seen in **Table 3.1**. The degree of substitution was determined using both ^{1}H NMR and FTIR by members of the Pasch group working on this study previously.[5]

Table 3.1 Description of the samples.

Sample code	L'Oréal code	DS (SU)	DS (L'Oréal)
HA 01	DGA1340144 (79544)	-	
HA 02	DGA1350354 (79544)	-	
HA 03	ES83501190 (79544)	-	
HAM 01	R0076848 A001 L002	3.1	2.4
HAM 02	R0076848 A002 L001	3.6	2.45
HAM 03	R0076848 A001 L003	2.9	2.53
HAM 04	R0076848 A003 L001	2.6	
HAM 05	R0077309 A001 L001	2.2	1.96
HAM 06	R0077308 A001 L001	0.8	-
HAM 07	R0077310 A001 L001	0.4	0.50
HA 1	HA 1	-	-
HA 2	HA 2	-	-
HAM 08	R0076848 A005 L002	2.6	2.04
HAM 09	R0076848 A005 L003	2.5	2.04
HAM 10	R0076848 A005 L004	1.5	1.68
HAM 11	R0076848 A005 L005	1.6	1.52

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3.3. Results and Discussion

3.3.1. Solubility Study

Polysaccharides such as HA are generally considered to be soluble in water.[8,9] The modification of the polar HA backbone through the hydroxyl groups with a hydrophobic acrylic moiety to various degrees reduced the water solubility of the HA polymer (see **Figure 3.1** and **Figure 3.2**).[11] The higher the DS, the more hydrophobic the HA polymer becomes, increasing the difficulty to dissolve the polymers in a hydrophilic environment.[5,11] Along with this, HA polymers tend to form aggregates when dissolution occurs in an aqueous medium, resulting in a decrease in solubility of the HA polymer (see **Figure 3.3**).[5,6]

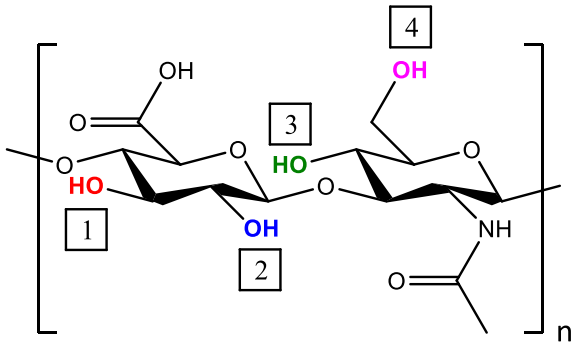

Figure 3.1 *Hyaluronic acid before modification of the hydroxyl groups as shown.*

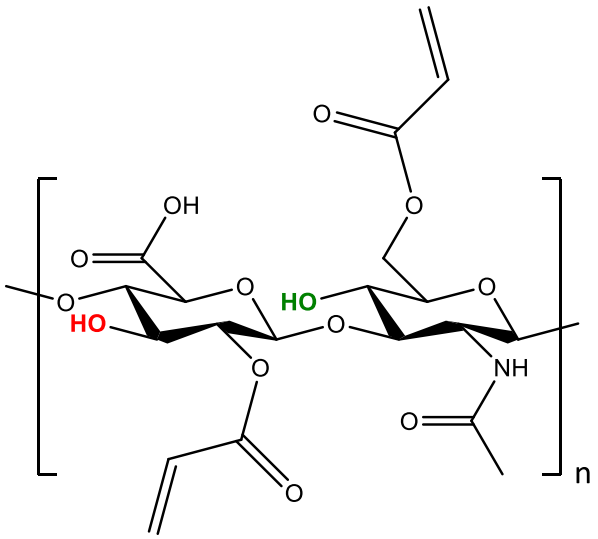

Figure 3.2 *Chemical structure of hyaluronic acid after modification of the hydroxyl groups with hydrophobic acrylate moieties (DS = 2).*

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Based on the results and insight gained from the comprehensive solubility study previously conducted on the HA polymers,[5] it was found that besides the DMSO:H_2O solvent system, 1,4-dioxane:H_2O and ACN:H_2O showed promise to dissolve the HA samples. The continuation of the solubility study mainly concentrated on finding a volatile salt that was compatible with an ELSD, to be used in conjunction with the solvent systems. The use of a volatile salt was to assist in the dissolution of the unmodified HA and modified HA samples irrespective of DS, as well as to optimize the gradient HPLC method and SEC method that were intended to be used in the first and second dimension. The volatile salts investigated are listed in **Table 3.2**.

Table 3.2 ELSD compatible salts and acids tested.

1. Ammonium acetate	2. Ammonium formate
3. Ammonium trifluoroacetate	4. Formic acid
5. Trifluoroacetic acid	

The selected volatile salt had to meet the following requirements, (1) be compatible with the available ELSD setup, (2) not degrade the sample and (3) be compatible with the separation column. When considering a volatile salt for analysis, the limitations of the ELSD detector should be taken into account. The ELSD (Agilent 1260 infinity) used for this study, had a maximum operating temperature of 100 °C. Thus, when investigating possible volatile salts, the evaporation temperature of the salt should be taken into account. The ELSD must be able to evaporate the salt so that it does not interfere with the polymer sample signal. Secondly, when the electrolyte is added to the given solvent system, the pH of the solvent system should remain neutral as a too basic or a too acidic environment can degrade the HA polymer and also degrade the analytical column. The pH of the solvent system had to be between 5 and 11, as it was found that HA could degrade below and above the respective pH range.[12,13] HA can undergo acid or alkaline hydrolysis and degrade.[12,13] In addition, the pH limitation of the separation column should be considered. The pH limitation of the separation column was between a pH of 2 and 12. The pH of the solvent systems could be manipulated by the salt concentration.

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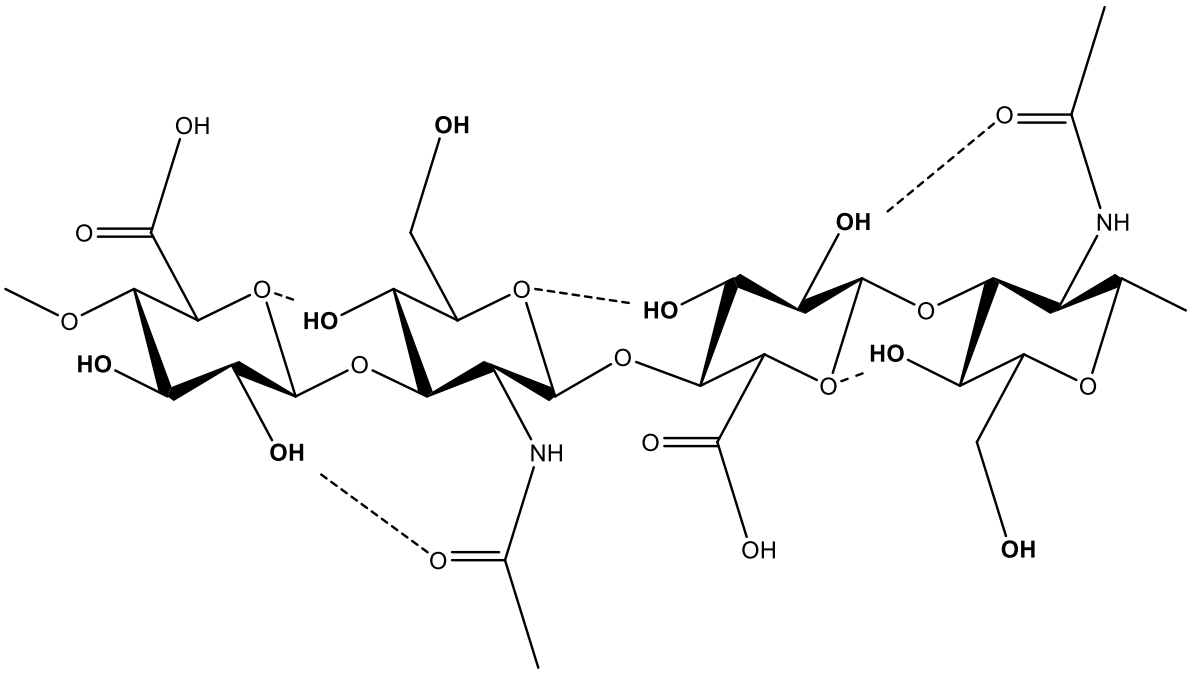

Figure 3.3 *Illustration of hydrogen bonding network that is present in hyaluronic acid.*

The solvent systems which showed promising results are listed in **Table 3.3** (see also **Appendix A** for detailed solubility results). The solubility of the HA polymers in the various tested solvent systems was determined by visual examination. Visual examination effectively means that the solubility results were obtained by simply looking at the samples after the dissolution period was complete at the predetermined temperatures and determining if the sample was dissolved or not. If the solution was clear, the sample was assumed to be dissolved and the solvent system was classified as a good system. During the course of the study it was observed that with an increase in temperature from 25 °C to 40 °C, the solubility of the HA polymers improved. For this reason, all further analyses were carried out at 40 °C. Additionally, it was found with the addition of a salt in low concentration to the solvent system, the solubility of the HA samples improved.

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Table 3.3 Favourable solvent systems for the dissolution of HA polymers based on visual examination.

1, 4 Dioxane:H_2O (40:60, v/v %) with 0.02 M Ammonium acetate ($NH_4CH_3CO_2$)	Acetonitrile:H_2O (40:60, v/v %) with 0.02 M Ammonium acetate ($NH_4CH_3CO_2$)
1, 4 Dioxane:H_2O (40:60, v/v %) with0.02 M Formic acid (CH_2O_2)	Acetonitrile:H_2O (40:60, v/v %) with 0.02 M Ammonium formate (NH_4HCO_2)

3.3.2. Dynamic Light Scattering (DLS)

Visual examination of the solubility, although a rapid verification method for assessing dissolution, is inherently flawed due to the limitations of minimum particle size detection. In such circumstances, a sample may appear to be completely dissolved; however, micron- and submicron-sized aggregates may still be present, which are undetectable by the eye.[14]

As an alternative to visual examination, dynamic light scattering was utilized. This had a two-fold benefit for determining a suitable solvent system for the given HA polymers. Firstly, the aggregate formation would immediately be made apparent with very large particle sizes being detected, and secondly, a systematic approach could be used to determine a suitable solvent system for the analytes. With the aid of DLS, the hydrodynamic size of the analytes in solution was determined. When a polymer chain is in a poor solvent system, the coil contracts to minimize unfavourable contact with the solvent molecules, resulting in small hydrodynamic sizes.[14] As the solvent system becomes more favourable for the given polymer, the coil expands into a random coil formation, resulting in larger hydrodynamic sizes.[14] By comparing the hydrodynamic sizes of the samples in various solvent systems with and without salt added, a suitable solvent system could be determined. The solvent systems that were most suitable for the dissolution of the HA polymers were analysed by DLS.

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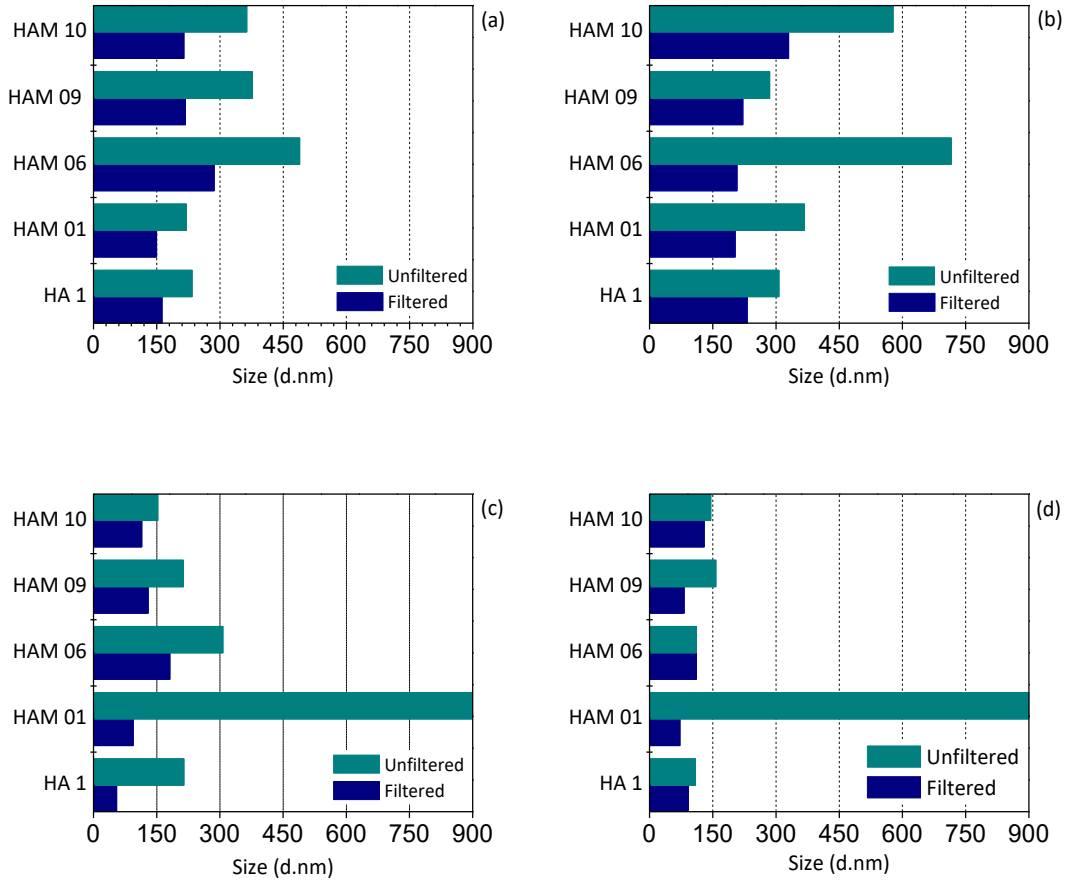

Figure 3.4 *Comparison of the size of the unmodified and modified HA (DS = 0 – 4) in solution before and after filtration through a 0.45 µm regenerated cellulose filter. (a) 1,4 Dioxane:H_2O (40:60, vol. %) with 0.02 M ammonium acetate (b) 1,4 Dioxane:H_2O (40:60, vol. %) with 0.02 M formic acid, (c) ACN:H_2O (40:60, vol. %) with 0.02 M ammonium formate, (d) ACN:H_2O (40:60, vol. %) with 0.02 M ammonium acetate.*

The results obtained emphasize the importance of incorporating DLS measurements in the process of finding a suitable solvent system for the HA polymers. HA is a complex polymer, in that it forms aggregates.[5,6] The size determined by DLS for the filtered HA polymer are in the range of 100 to 300 nm. The size of a single macromolecule is expected to be in the range of 100 nm. Thus, it is clear from the results that there is still aggregate formation present in the sample solution and that single and isolated macromolecules are frequently not obtained. With the aid of DLS, a true representation of the solubility can be obtained compared to sample solubility determined by visual examination. Therefore, it is important to compare the unfiltered HA polymer with the filtered HA polymer. In the case of visual

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examination, if a sample can be filtered, it is assumed that the sample is properly dissolved. However, it was apparent from the DLS results that upon filtration, larger aggregates could be lost, which would not be evident by simple visual examination and filtration. If a significant difference between the unfiltered and filtered samples is observed, it effectively indicates that the larger aggregates are removed, which means that the aggregates are present. It is evident from the results in **Figure 3.4 (a)** and **(b)**, that for the HA polymers dissolved in 1,4-dioxane:H_2O aggregates are present to a large degree compared to the ACN:H_2O solvent systems (see **Figure 3.4 (c)** and **(d)**). Thus, with DLS it can be quantitatively determined what really is being analysed. From the DLS results, it was concluded that the ACN:H_2O solvent systems with the different volatile salts are better solvent systems for the HA polymers, as there was minimal difference in size between the unfiltered and filtered samples. The sizes determined were in a reasonable range of 150 nm. However, it was observed that the ACN:H_2O solvent system was not favourable for sample HAM01. A possible reason for this could be that HAM01 was modified with the acrylic moiety to a higher degree compared to the other samples, making the molecules very hydrophobic.

3.4. Conclusion

Based on the solubility studies conducted it was found that with the addition of a volatile salt, a suitable solvent system could be obtained that was able to dissolve the HA polymers over the range of DS = 0 - 4. The salt assisted in the dissolution process as it was observed that there was an improvement in the solubility of the HA polymers in the given solvent systems. With the aid of DLS, a true representation could be obtained of how the samples behave in the various solvent systems. The presence of aggregates could be determined that are inherent to HA polymers. DLS, thus, assisted in obtaining quantitative information on the HA polymer with regard to what exactly would be subjected to LC analysis.

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3.5. References

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Chapter 4

Size Exclusion Chromatography Method Development

This chapter will focus on the molar mass determination of the unmodified HA as well as the modified HA samples by size exclusion chromatography (SEC). Emphasis will be placed on the optimization of the mobile phase with the addition of a volatile salt.

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4.1. Introduction

The modification of the HA results in a complex polymer that is heterogeneous with regard to chemical composition distribution (CCD) and molar mass distribution (MMD). The structural modification influences the properties of the polymer.[1-3] Therefore, it is essential to characterize these distributions in order to establish a reliable relationship between the structures and the properties of the polymer samples.[1-4] Liquid chromatography techniques such as SEC is well suited for analysing the heterogeneity of the polymers with regard to molar mass.[4-6] However, the characterization of the HA samples by SEC can be difficult due to the inherent solubility and stability challenges of HA. Modification of the hydrophilic HA backbone with hydrophobic acrylate moieties influences the solubility of the HA.[7-9] With the addition of the acrylate moiety, the polarity of the polymer is reduced and thus determines the LC method and stationary phase that would be used for characterization. In addition to the modification giving rise to solubility problems, HA samples form non-covalent aggregates with each other in solution. As a result, the hydrodynamic volume of the apparent polymer increases and the polymer aggregates will elute at a lower SEC elution volume, thus appearing to be larger in molecular size than they truly are. It is, therefore, necessary to select a suitable solvent system and mobile phase for SEC analysis that should not only dissolve the macromolecules but also assist in reducing aggregate formation in order to determine correct average molar masses. Another essential criterion for the developed SEC method is that it should be compatible with an ELSD detector as the developed method would subsequently be used as the second dimension in 2D-LC.

4.2. Experimental and Instrumentation

4.2.1. Solvents and Chemicals

Acetonitrile (ACN) (HPLC grade, Sigma-Aldrich), water (H_2O) (Millipore from laboratory H_2O filtration system), ammonium acetate ($NH_4CH_3CO_2$) (Sigma-Aldrich), ammonium formate (NH_4HCO_2) (Sigma-Aldrich) and ammonium trifluoracetate ($NH_4CF_3CO_2$) (Sigma-Aldrich). The solvents and chemicals were used as received.

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4.2.2. Size exclusion chromatography coupled with a differential refractive index detector (dRI) and an evaporative light scattering detector (ELSD)

SEC analysis was performed on an Agilent 1200 series instrument equipped with a vacuum degasser, a quaternary pump, an autosampler, column oven, an Agilent 1100 differential refractive index (dRI) detector and an Agilent 1260 infinity evaporative light scattering detector (ELSD). The column oven temperature was kept at 40 °C and 100 µL of 1.0 $mg{\cdot}mL^{-1}$ sample was injected into the column set that consisted of PSS-Suprema 1000 Å (300 mm x 8 mm i.d.) and Suprema 30 Å (300 mm x 8 mm i.d.) columns, both with a particle size of 10 µm in series and protected with a Suprema guard column. A flow rate of 1.0 $mL{\cdot}min^{-1}$ was used for analysis. The detector temperature for the dRI was set at 40 °C. The ELSD was set to a nebulization temperature of 100℃ and the sensitivity (gain) of the detector to 6 mV. The system was calibrated using narrow poly(ethylene oxide) standards ranging from 900 to 1,000,000 $g{\cdot}mol^{-1}$. Data acquisition and calibration were done with WinGPC Unity software (version 7, PSS Polymer Standards Service GmbH, Mainz, Germany). All data processing was done with OriginPro 8.0.

4.2.3. Sample preparation

Samples with a concentration of 1.0 $mg.mL^{-1}$ were prepared by dissolving 2.0 mg of sample in 2.0 mL of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate, stirred for 20 hours at 40 °C at 500 rpm. Before analysis, the samples were filtered through a 0.45 µm regenerated cellulose filter. The samples were kept in the absence of light during both the dissolution process and the analysis of the samples. This minimized the exposure to light as UV radiation may lead to sample degradation.[10]

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4.3. Results and discussion.

4.3.1. Influence of salt on the elution behaviour of modified HA.

SEC of amphiphilic analytes, such as HA samples, very frequently requires the addition of an electrolyte to the mobile phase. These analytes tend to form aggregates even in thermodynamically good solvents and the addition of a salt reduces aggregate formation. In order to characterize the unmodified HA and modified HA (HAM) according to molar mass distribution, a volatile salt was added to both the sample solvent and the mobile phase as HA samples have a tendency to form aggregates in solution due to non-covalent interactions.[5,6] A volatile salt was required as an ELSD was to be used in the SEC method which will also be used as the primary detector in 2D-LC. In addition to the ELSD, the conventional dRI detector was used.

Based on the DLS results, the ACN:H_2O (40:60, vol.%) solvent system with different volatile salts was investigated as possible mobile phase for LC. The effect of the salt on the elution behaviour of the polymers was investigated first. The samples were dissolved in ACN:H_2O (40:60, vol.%) and ACN:H_2O (40:60, vol.%) with ammonium acetate, respectively, and analysed using an aqueous PSS Suprema column set. The column was chosen as the solvent system predominantly consisted of water and as it is well suited for characterizing HA polymers. **Figure 4.1** illustrates the influence of the added salt on the elution behaviour of the representative HA samples (HA 1 (DS = 0); HAM 01 (DS= 3.1); HAM 06 (DS = 0.8) and HAM 09 (DS = 2.5)). It is evident from the results that the addition of a volatile salt assisted in reducing the aggregate formation of the HA samples when characterized by SEC, as the samples eluted within the separation range of the column. The exclusion limit of the column was 13.26 mL, as established with PEO calibration standards.

After it was established that the addition of a volatile salt improved the separation, different salts were tested. The tested salts included ammonium acetate, ammonium formate and ammonium trifluoroacetate. The experiments were performed in order to investigate if the different salts reduced the aggregation formation for the selected HA samples (HAM 01 (DS= 3.1); HAM 06 (DS = 0.8), HAM 09 (DS = 2.5) and HAM 10 (DS = 1.5)) to different degrees. The results are shown in **Figure 4.2**.

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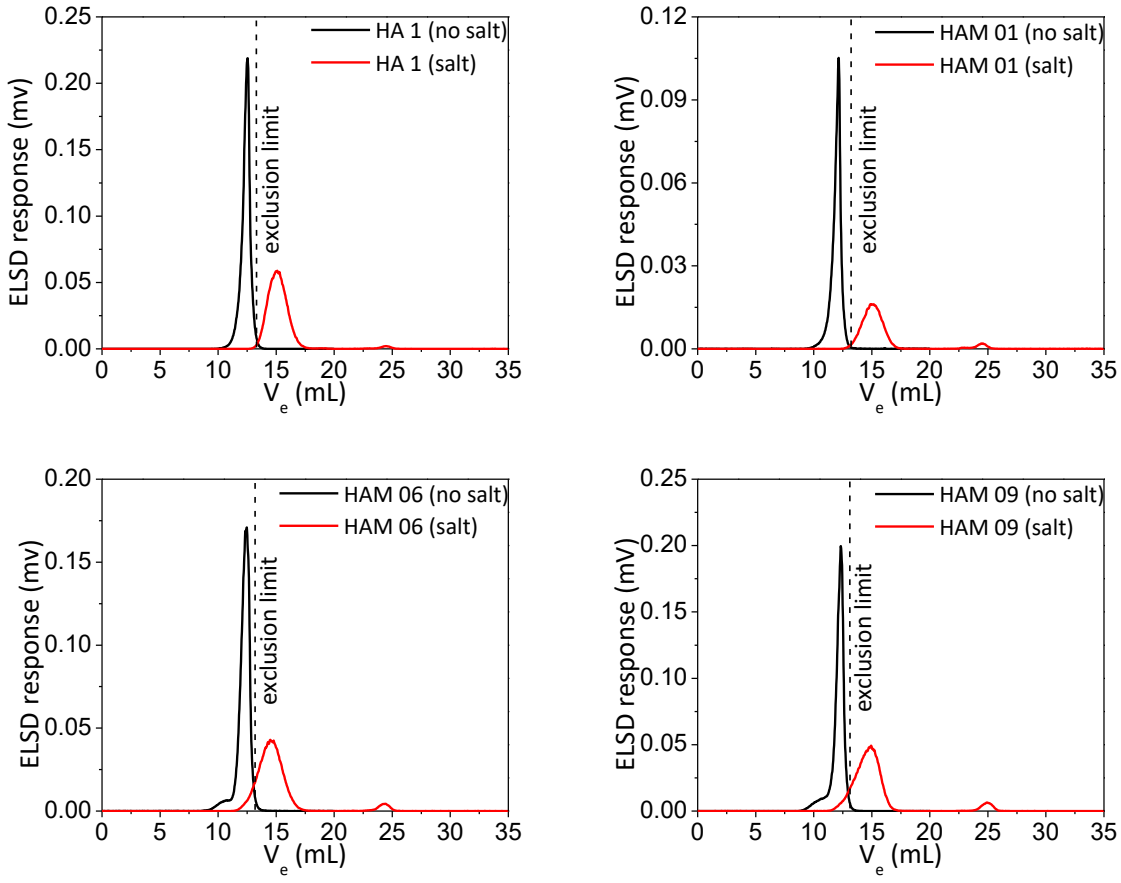

Figure 4.1 *Overlay of SEC chromatograms of HA samples varying in average DS values (DS = 0 – 3.1) illustrating the influence of ammonium acetate on the elution behaviour of the polymers. Stationary phase: PSS-SUPREMA (guard, 1000 Å + 30 Å; 300 mm x 8.0 mm I.D. 10 µm); mobile phase: ACN:H_2O (40:60 vol. %) (**black**), ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate (**red**). Sample solvent: ACN:H_2O (40:60 vol. %) (**black**), ACN:H_2O (40:60 vol %) with 0.02 M ammonium acetate (**red**) The exclusion limit of the calibration: 13.26 mL .*

It was evident from the results obtained, that the type of salt had no influence on the elution behaviour of the HA polymers. In addition, it was established that all the tested salts did reduce the formation of aggregates in the HA samples. For ammonium acetate and ammonium trifluoroacetate, a small peak elutes between 25 to 30 mL. The eluting peak is outside the calibration limit, which was determined to be 19.16 mL (PEO standard, 982 $g.mol^{-1}$). The small eluting peak observed in the elugrams could be attributed to the volatile salt as a small salt peak will always be expected regardless of being volatile or not.

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Ammonium acetate and ammonium trifluoroacetate have evaporation temperatures of 114 °C and 123 °C, respectively and could possibly not be evaporated completely by the ELSD, which has a maximum operating temperature of 100 °C. The sensitivity of the ELSD is extremely high, thus even if a small amount of salt is not evaporated, the salt can be detected. This phenomenon requires further investigation, as the eluting behaviour of the last eluting peak was not consistent for all samples. However, the mobile phase in each case is different due to the different salt that was added to the ACN:H_2O (40:60, vol.%) solvent system. Thus, with each mobile phase, the random coil formation of the polymer will change, hence the reason for variation observed in the elution behaviour of the last eluting peak.

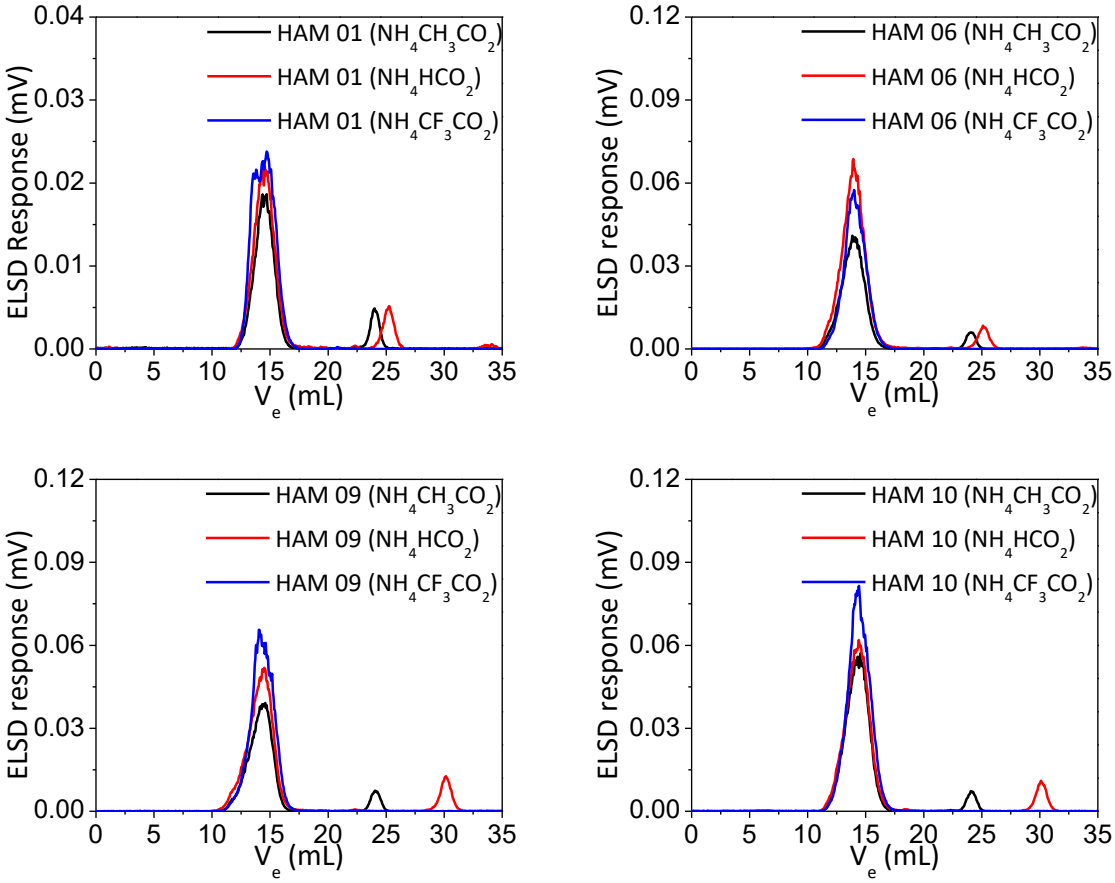

Figure 4.2 *Overlay of SEC chromatograms of HA samples (DS = 0 – 3.1) demonstrating the influence of the type of salts on the elution behaviour of the polymers. Mobile phase and sample solvent: ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate* ***(black)****, ACN:H_2O (40:60 vol. %) with 0.02 M ammonium formate* ***(red)****, ACN:H_2O (40:60 vol. %) with 0.02 M ammonium trifluoroacetate* ***(blue)****.*

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As the next step in the development of the SEC method, the influence of the salt concentration on both the elution behaviour and on minimization of the aggregate formation was investigated. Salt concentrations of 0.02 M and 0.10 M ammonium acetate were tested. Ammonium acetate was selected based on the DLS results (Chapter 3) obtained, which indicated that this salt aided best in reducing the aggregate formation. From the results presented in **Figure 4.3**, it was observed that the samples dissolved with the higher salt concentration sample solvent and separated with a mobile phase with higher salt concentration, had slightly broader eluting peaks, while the samples dissolved in lower salt concentrations, had a narrower eluting peak. The small late eluting peak also increased with an increase in salt concentration. This supports the assumption previously stated, that the salt is not entirely evaporated by the ELSD during analysis. It was also observed that the last eluting peak shifted towards a later elution volume. This can be attributed to the fact that the mobile phase in each case is different due to the different salt concentration used. As the mobile phase and sample solvent are different, the random coil conformation of the polymer chain will change, and as expected, if the random coil conformation change, the hydrodynamic volume change. As a result, broadening of the eluting peak or shift in elution would be observed. Thus, for further analysis the 0.02 M salt concentration was used as it was concluded to be best suited for determining the molar mass distribution of the HA samples.

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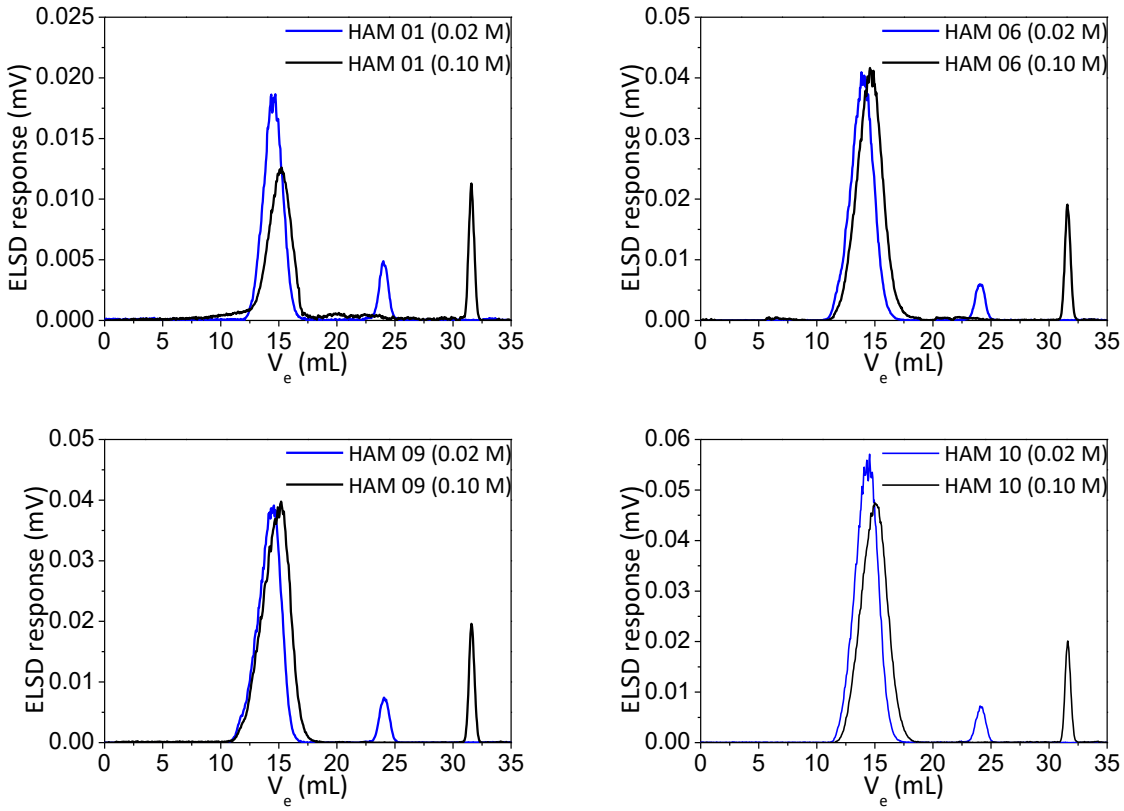

Figure 4.3 *Overlay of SEC chromatograms of HA samples varying in average DS values* (HAM 01 (DS= 3.1); HAM 06 (DS = 0.8), HAM 09 (DS = 2.5) and HAM 10 (DS = 1.5)) *illustrating the influence of salt concentration on the elution behaviour of the polymers. Stationary phase: PSS-SUPREMA (guard, 1000 Å + 30 Å; 300 mm x 8.0 mm I.D. 10 µm). Mobile phase and sample solvent: ACN:H_2O (40:60 vol. %) with x M ammonium acetate, 0.02 M (**blue**) and 0.10 M (**black**).*

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4.3.2. Molar mass determination of modified HA.

The characterization of the HA samples with regards to molar mass distribution was determined by using a dRI and ELSD in series, which were calibrated using PEO calibration standards (see **Table 4.1**). The standards were dissolved in ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate, the same solvent system as the HA samples. It should be noted that the molar masses determined by SEC are not an absolute representation of the molar masses of the HA polymers as the chemical composition of the HA polymers is significantly different to that of the PEO calibration standards. Therefore, the molar masses determined have an inherent error associated with the calibration and for that reason, the molar masses are referred to as average values relative to the PEO calibration. The difference observed in the molar mass determined by dRI and ELSD, respectively, can be attributed to the fact that different to that of ELSD, dRI has a linear concentration dependency.

Table 4.1 Molar masses (PEO equivalents) and molar mass dispersities of HA samples

		dRI[a]			ELSD[a]		
Sample Code	**DS**	**M_n ($g.mol^{-1}$)**	**M_w ($g.mol^{-1}$)**	**Đ**	**M_n ($g.mol^{-1}$)**	**M_w ($g.mol^{-1}$)**	**Đ**
HA 1	0	36900	119700	3.25	76000	179200	2.36
HAM 01	3.1	58800	145300	2.47	83600	205200	2.46
HAM 03	2.9	61700	240200	3.89	95000	308000	3.24
HAM 04	2.6	69600	292100	4.20	87000	351600	4.04
HAM 05	2.2	63100	157300	2.49	77900	208000	2.67
HAM 06	0.8	81000	344600	4.25	127600	442800	3.47
HAM 07	0.4	89800	249700	2.78	165000	457400	2.77
HAM 08	2.6	60100	169400	2.82	96000	261900	2.72
HAM 09	2.5	82100	287600	3.50	133300	448600	3.36
HAM 10	1.5	65400	243700	3.73	113900	388400	3.41
HAM 11	1.6	64300	232800	3.62	107600	346440	3.22

a. PEO calibration.

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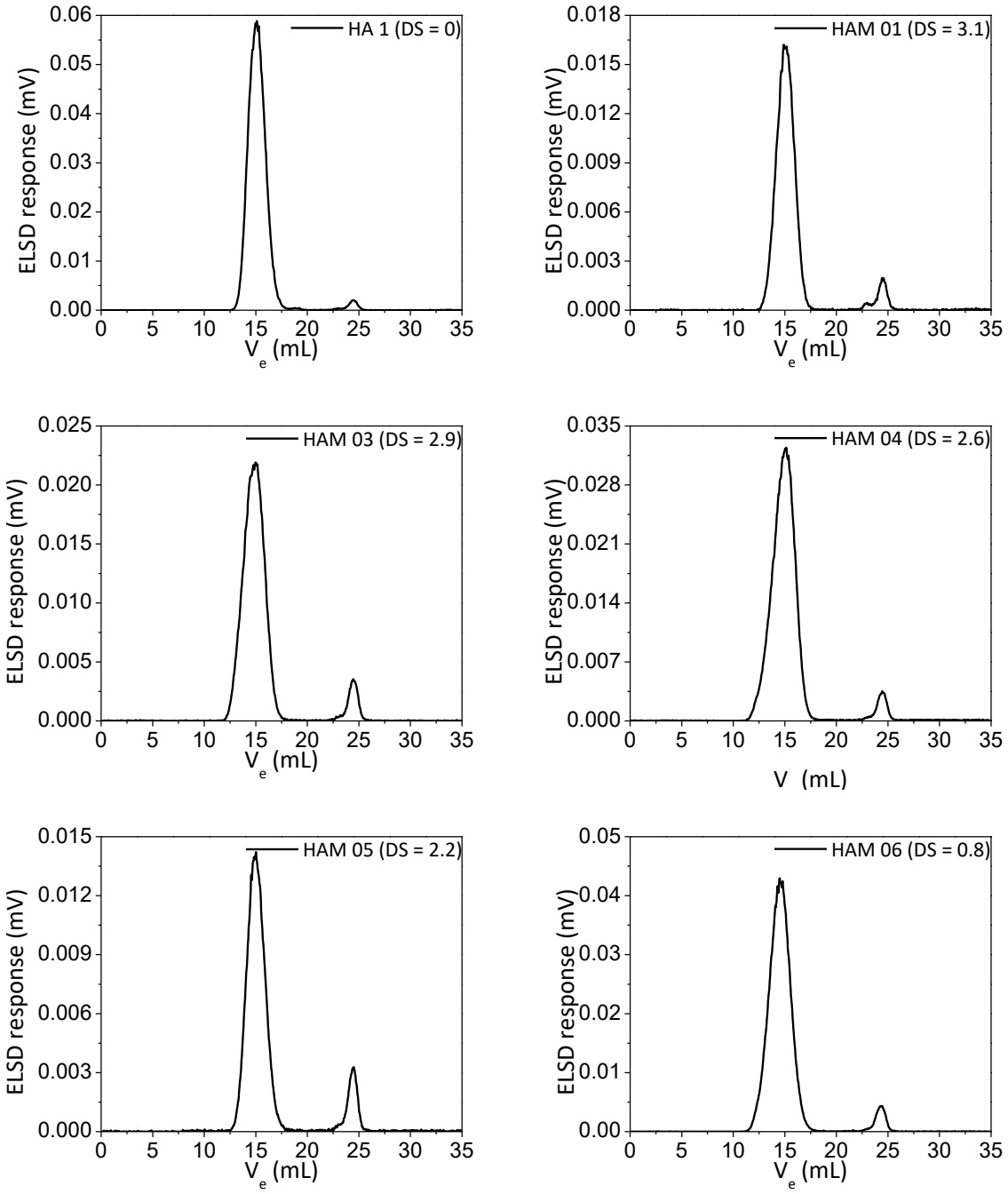
Figure 4.4 shows the ELSD traces of the individual SEC chromatograms of HA samples with varying DS (DS = 0 to 3.1). It was evident from the elugrams that a unimodal distribution for each of the samples was obtained, which suggested that ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate used for sample dissolution as well as the mobile phase eluent, was well suited. However, some samples started to elute close to the exclusion limit for the column set, 13.26 mL, which could indicate that non-covalent aggregation could still be occurring to some extent. It is assumed, however, that this effect is very low.



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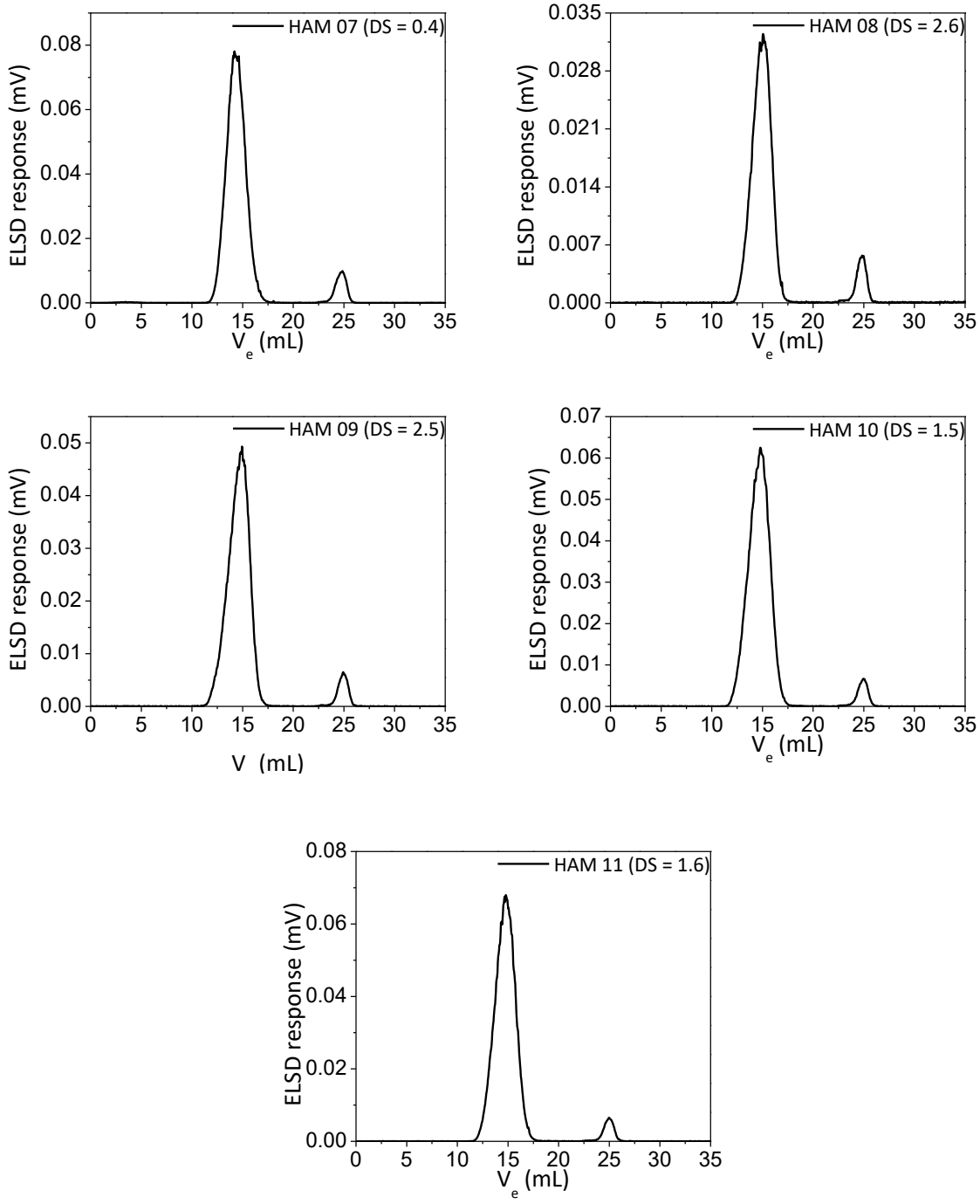

Figure 4.4 *ELSD chromatograms of HA samples varying in average DS values (DS = 0 – 3.1) Stationary phase: PSS-SUPREMA (guard, 1000 Å + 30 Å; 300 mm x 8.0 mm I.D. 10 µm); mobile phase: ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate, Sample solvent: ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate. The exclusion limit of the calibration: 13.26 mL.*

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4.4. Conclusion

A novel SEC method using a mobile phase of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate was developed for the molar mass characterization of the unmodified and modified HA in the range of DS = 0 to 3.1. It was found that with the addition of a salt the extent of aggregate formation was largely reduced and hence proper separation of the HA samples was obtained, as the samples eluted within the separation range of the columns in contrast to samples analysed without salt addition. Most importantly, the new SEC method was suitable for ELSD detection and hence for the intended 2D-LC setup for comprehensive chemical composition and molar mass analysis of the samples.

4.5. References

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Chapter 5

High Performance Liquid Chromatography Method Development

This chapter will focus on the development and optimization of a reversed phase liquid chromatography (RP-LC) method to separate hyaluronic acid derivatives according to their chemical composition. In addition to the RP-LC method development, this section will focus on the characterization of the chemical composition of the various HA derivatives as well as a brief investigation into the possibility of sample degradation over the course of this study.

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5.1. Introduction

HA and acrylate-modified HA (HAM), are intrinsically complex polymers which require the development of advanced analytical techniques for comprehensive characterization. One such method is high performance liquid chromatography (HPLC) which allows for the separation according to chemical composition, which is the degree of acrylate substitution (DS) of HA. Predominantly, solvent or temperature-gradient liquid chromatography (LC) is used for the separation of heterogeneous complex polymers.[1-3] HPLC method development comprises five main steps:

1. Determination of the most suitable mode of separation.
2. Determination of the best suitable chromatographic conditions.
3. Selectivity and resolution optimization.
4. Chromatographic conditions optimization.
5. Method verification.

For the separation of the unmodified and modified HA, reversed phase liquid chromatography (RP-LC) was investigated as a potentially improved mode of separation, as previous studies were conducted with normal phase LC.[4] With RP-LC, the stationary phase is non-polar, typically C-8 or C-18, which results in analyte separation according to hydrophobicity.[5,6] This order of separation is based on the affinity that various non-polar heterogeneous analytes have towards the stationary phase.[5] With gradient RP-LC, the mobile phase initially consists of a polar solvent (thermodynamically unfavourable for the analyte) and over time the polarity of the solvent system is decreased to a more thermodynamically favourable solvent system for the analyte.[7] Thus, during gradient LC, the injected sample, which was dissolved in a thermodynamically favourable solvent system, is subjected to a poor dissolution mobile phase, resulting in precipitation or adsorption with little to no separation. As the gradient proceeds towards a more non-polar eluent, the analyte molecules begin to have a greater affinity for the mobile phase and thus separation occurs according to the degree of non-polar nature of the analyte, or in other words, chemical composition.[5,6] The overall result is that polar analytes elute before non-polar analytes as there is little retention of the polar analytes in a non-polar stationary phase. In

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this investigation, the HA samples, which are polar in nature, were modified to various extents with non-polar moieties (acrylate groups).

The modification of the HA samples occurred at the hydroxyl functionalities by substituting the hydroxyl with acrylate moieties. The introduction of acrylate groups reduces the polarity of the sample, and thus the sample becomes more non-polar. Thus the separation of the polymer samples according to chemical composition can be achieved through the use of RP-LC.

5.2. Experimental and Instrumentation

5.2.1. Solvents and Chemicals

Acetonitrile (ACN) (HPLC grade, Sigma-Aldrich), dimethyl sulfoxide (DMSO) (HPLC grade, Sigma Aldrich), water (H_2O) (Millipore from laboratory H_2O filtration system), ammonium acetate ($NH_4CH_3CO_2$) (Sigma-Aldrich) and deuterium oxide (D_2O) (Merck). The solvents and chemicals were used as received.

5.2.2. High performance liquid chromatography coupled with an ELSD

All HPLC analyses were performed on an Agilent 1200 series instrument equipped with a vacuum degasser, quaternary pump, autosampler, column oven, and an Agilent 1260 Infinity evaporative light scattering detector (ELSD). Data acquisition was done with WinGPC Unity software (version 7, PSS Polymer Standards Service GmbH, Mainz, Germany) and data processing was done with OriginPro 8.0. The separation was carried out on an Agilent Zorbax C8-RX column (150 × 2.1 mm i.d.) with 5 µm average particle size. The linear gradient mobile phase comprised initially of H_2O (100 vol. %) to a composition of ACN:H_2O (45:55 vol. %) at a flow rate of 0.5 mL.min^{-1}. The mobile phase for step-wise gradient HPLC consisted of 100 % H_2O to 50:50 vol. % ACN: H_2O at a flow rate of 0.5 mL·min^{-1}. The oven temperature was kept at 40 °C for all analyses. An injection volume of 30 µL was used for all analyses. The ELSD was set to an operating temperature of 100 °C, with the sensitivity (gain) set to 6 mV. For fraction collection, an Agilent 1200 automated fraction collector was used.

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5.2.3. Sample preparation

All samples were prepared by dissolving 2.0 mg polymer in 2.0 mL of solvent to obtain a sample concentration of 1.0 $mg.mL^{-1}$, unless otherwise stated. The dissolution solvent composition consisted of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate. The samples were stirred for 20 hours at 40 °C at 500 rpm in the absence of light and filtered through a 0.45 µm regenerated cellulose filter before analysis.

Note: During the dissolution process the samples were covered with aluminium foil and during all analyses, the samples were kept in the absence of light to minimise sample degradation due to UV radiation.[8]

5.2.4. Void volume determination

Table 5.1 Void volume

Void volume of column	0.3 mL
Void volume of system	1.1 mL

The void volume of the chosen column was determined theoretically and experimentally. This is of importance as an analyte which elutes near or at the void volume indicates that little to no chromatographic interaction occurred. The void volume was determined theoretically to be 0.3 mL by using the following equation:

$$\text{Void volume } (\mu L) = (d^2 \times \pi \times L * 0.7)/4$$

Where *d* is the column diameter and *L* the column length, in millimetres and 0.7 is the standard pore volume. It is generally assumed that the average pore volume is 70% (i.e. 0.7) for a packed column.

Experimentally, the void volume of a column is determined by injecting an unretained non-volatile compound. Sodium nitrite was chosen as the non-volatile compound as it is not retained under the chosen conditions and will not be evaporated by the universal ELSD. Sodium nitrite was dissolved in water and firstly injected onto the column under isocratic

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conditions with water as the mobile phase and then injected without a column (i.e. capillary) to determine the system void volume. The system void volume refers to any additional dead volume contributed by connections to the column and from the column to the detector. The column void volume is therefore determined by the difference between the elution volume of sodium nitrite with a column and the system void volume. The experimental void volume for the column was determined to be 0.3 mL, which is in agreement with the theoretical void volume.

5.3. Results and discussion

5.3.1. Linear gradient

To investigate the elution behaviour of HA and modified HA samples, reversed phase C-18 and C-8 columns were used in the subsequent investigation. The main focus was to develop a gradient HPLC method which has the ability to not only separate the unmodified polar HA from the modified non-polar HA, but also to separate according to the different degrees of substitution i.e. chemical composition.

A linear gradient of H_2O (100 vol. %) to ACN:H_2O (50:50 vol. %) was performed on both the C-18 and C-8 columns. ACN and H_2O were chosen as the mobile phase based on the findings of the previous solubility studies conducted (see **Chapter 3**). It was concluded that the most suitable solvent system for all HA samples, modified and unmodified, irrespective of DS, consisted of ACN:H_2O (40:60 vol. %) with ammonium acetate as a salt. The individual LC methods developed and solvent systems chosen must be compatible with the available ELSD setup which will later be incorporated into a 2D-LC setup. The unmodified and modified HA was successfully separated according to the DS utilizing a linear mobile phase gradient (**Figure 5.1**). The acrylate functionality of the HA samples had a significant effect on the elution behaviour of the polymers, as a distinct difference in elution behaviour was observed. The acrylate groups reduce the polarity of the samples.[4] HA substituted to a higher degree with the acrylate moieties, will become less polar and retained longer on the non-polar RP column.[4]

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In addition, recovery tests were performed on the columns under isocratic conditions to determine the most suitable column for further analysis of the various HA samples.[9,10] The mobile phase composition at the point where the analyte elutes from the column while the gradient is applied is known to be near the critical condition of the analytes.[11] This means that at that at the specific solvent combination, the analyte will elute at that specific volume under isocratic conditions. The isocratic conditions chosen for the recovery tests were ACN:H_2O (50:50 vol. %). The recovery tests were done by injecting the sample and comparing the peak area obtained with and without the column (i.e. just a capillary). Based on the recovery tests, elution profiles and the resolution, it was concluded that the C-8 column was more suited for the characterization of the modified and unmodified HA samples than the C-18 column.

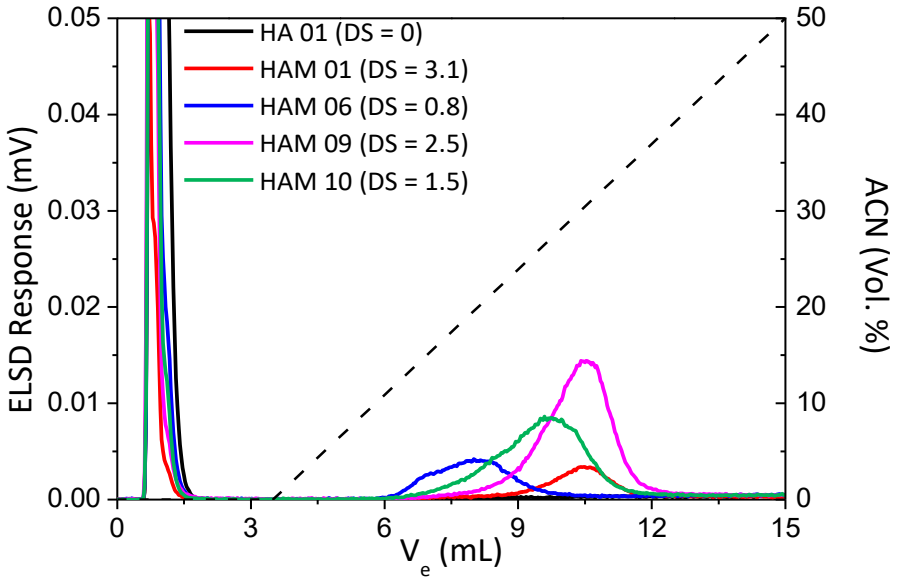

Figure 5.1 *Overlay of linear gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0 – 3.1). Stationary phase: Agilent Zorbax C8-RX (150 × 2.1 mm i.d.) with 5 µm particle size; mobile phase: H_2O (100 vol. %) to ACN:H_2O (50:50 vol. %).*

To achieve optimum selectivity and separation according to the DS of different HA samples on the C-8 column, different sample solvent compositions were investigated. The linear gradient used for the analysis is shown in **Figure 5.2 (a)** and was modified from the initial

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gradient used for the comparative C-8 and C-18 analysis The (linear gradient) mobile phase comprised initially of H_2O (100 vol. %) and was then changed over time to an eluent composition of ACN:H_2O (45:55 vol. %). The various sample solvent systems investigated included (1) DMSO:H_2O (60:40 vol. %) (**Figure 5.2, (b)**), (2) ACN:H_2O (40:60 vol. %) (**Figure 5.2, (c)**) and (3) ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate (see **Figure 5.2, (d)**). For each of the solvent systems, the unmodified HA and the modified HA were successfully separated according to DS.

For samples dissolved in DMSO:H_2O (60:40 vol. %) and ACN:H_2O (40:60 vol. %), respectively, two distinct broad peaks were observed. The first peak at 1.5 mL was attributed to the unmodified HA or unretained modified HA (breakthrough peak) and the second peak was attributed to the modified HA. The breakthrough effect can occur when a polymer is dissolved in a thermodynamically good eluent and when injected is subjected to a sudden change in eluent strength. As a result, the polymer is not retained and elutes near or at the void volume of the column due.[12,13]

For the samples dissolved in the solvent consisting of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate, and separated with the chosen linear gradient, a tri-modal distribution was observed for samples modified to a high degree of substitution (HAM 10 and HAM 04), indicating that the substitution reaction is very heterogeneous and preferential. In the case of HAM 06 (DS = 0.8) only two peaks were observed and for HA 1 (DS = 0), only one eluting peak was observed. The first observed peak which eluted close to the void volume (1.1 mL) in 100 % H_2O, was assigned to the unmodified HA or due to the breakthrough effect. The second eluting peak (6 – 12 mL) was assigned to modified HA with a lower DS. The third eluting peak (12 – 17 mL), present in samples HAM 10 and HAM04, was assigned to modified HA with a higher DS. It is evident from the separation and elution profile obtained that the presence of a salt in the sample solvent enhanced the selectivity of the developed linear gradient method. The salt present in the ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate sample solvent, reduced the extent of aggregate formation, hence a bimodal distribution was observed between 6 - 17 mL, instead of one single broad peak as seen for samples dissolved in DMSO:H_2O (60:40 vol. %) and ACN:H_2O (40:60 vol. %).

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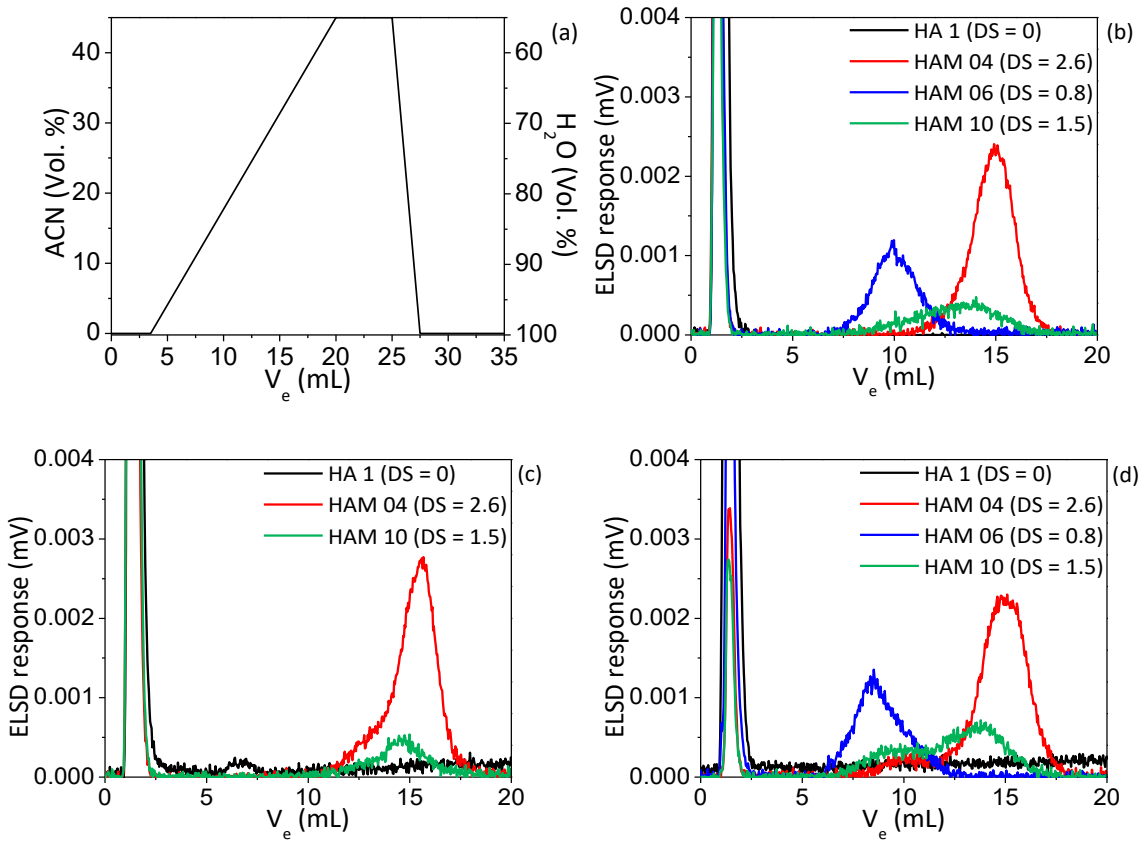

Figure 5.2 *(**a**) Linear gradient with the final composition of ACN:H_2O (45:55, vol. %), (**b-d**) elugrams illustrating the influence of different sample solvents on the elution behaviour of the unmodified and modified HA. Stationary phase: Agilent Zorbax C8-RX (150 × 2.1 mm i.d.) with 5 µm particle size; mobile phase: H_2O-ACN, Sample solvents: (**b**) DMSO:H_2O (60:40 vol. %), (**c**) ACN:H_2O (40:60 vol. %) and (**d**) ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate.*

5.3.2. Stepwise gradient

The linear gradient method developed in **Section 5.3.1** was able to separate the unmodified and modified HA according to average DS. The method was further optimized by utilizing a step-wise gradient as illustrated in **Figure 5.3**, with the distinct steps tabulated in **Table 5.2.**

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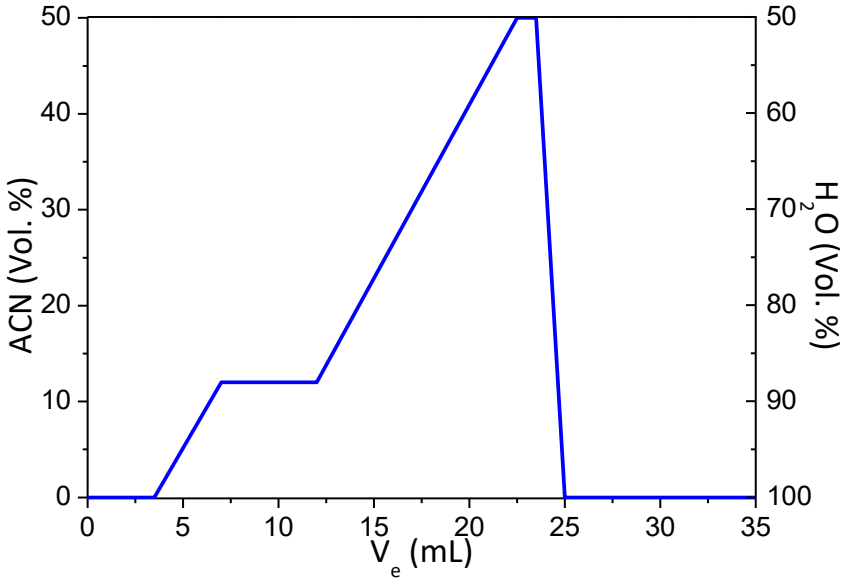

Figure 5.3 *Stepwise gradient elution profile used for the separation of the modified and unmodified hyaluronic acid with varying average DS (DS = 0 to DS = 3.1). Stationary phase: Agilent Zorbax C8-RX (150 × 2.1 mm i.d.) with 5 µm particle size; mobile phase: H_2O-ACN (see **Table 5.2** for stepwise gradient profile).*

Table 5.2 Stepwise Gradient Profile

V_e (mL)	**ACN (vol. %)**	**H_2O (vol. %)**
0	0	100
3.5	0	100
7	12	88
12	12	88
22.5	50	50
23.5	50	50
25	0	100
35	0	100

Baseline separation between the different chemically distinct components, within a given sample dissolved in ACN:H_2O (40:60 vol. %) with 0.2 M ammonium acetate and separated with the step-wise gradient method, was achieved. The results are illustrated in **Figure 5.4**.

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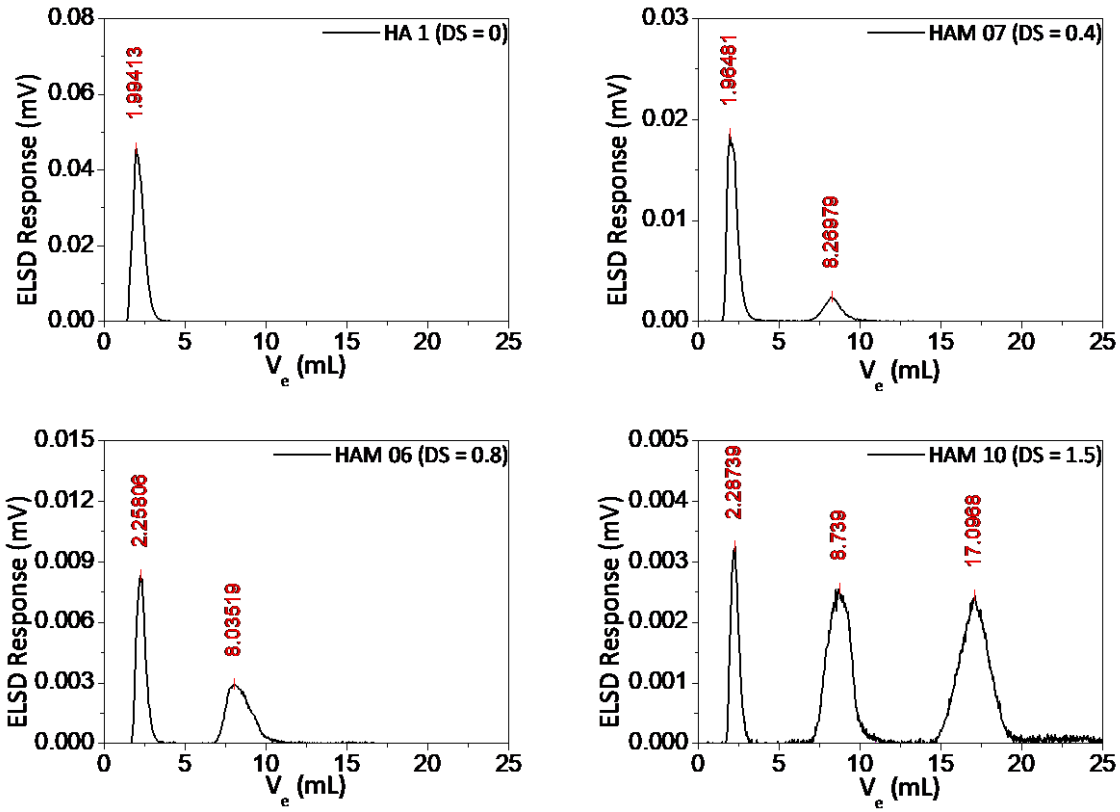
The elution profile for each of the higher DS HA samples (HAM 10 (DS = 1.5), HAM 11 (DS = 1.6), HAM 09 (DS = 2.5), HAM 04 (DS = 2.6), HAM 08 (DS = 2.6), HAM 03 (DS = 2.9) and HAM 01 (DS = 3.1)) revealed three distinct peaks, while the lower DS HA samples (HAM 07 (DS = 0.4) and HAM 06 (DS = 0.8)) contained two distinct peaks. Unmodified HA (HA 1 (DS = 0)) consisted of only one eluting peak. The first peak eluting at ±2 mL was assigned to unmodified HA or unretained modified HA, while the second (± 8 mL) and third peaks (±17 mL) were attributed to HA with increasing DS. It was noticed that the ratio of the signal of the second eluting peak relative to the last eluting peak changed with DS. The intensity of the last eluting peak increased in intensity, while the second eluting peak decreases with an increase in DS**.** It was evident from the chromatograms that each sample contains, to some extent, an unmodified component and various degrees of modified components. The chemical composition of each eluting peak was confirmed by ^{1}H NMR spectroscopy after fractionation (**Section 5.3.2.2**).



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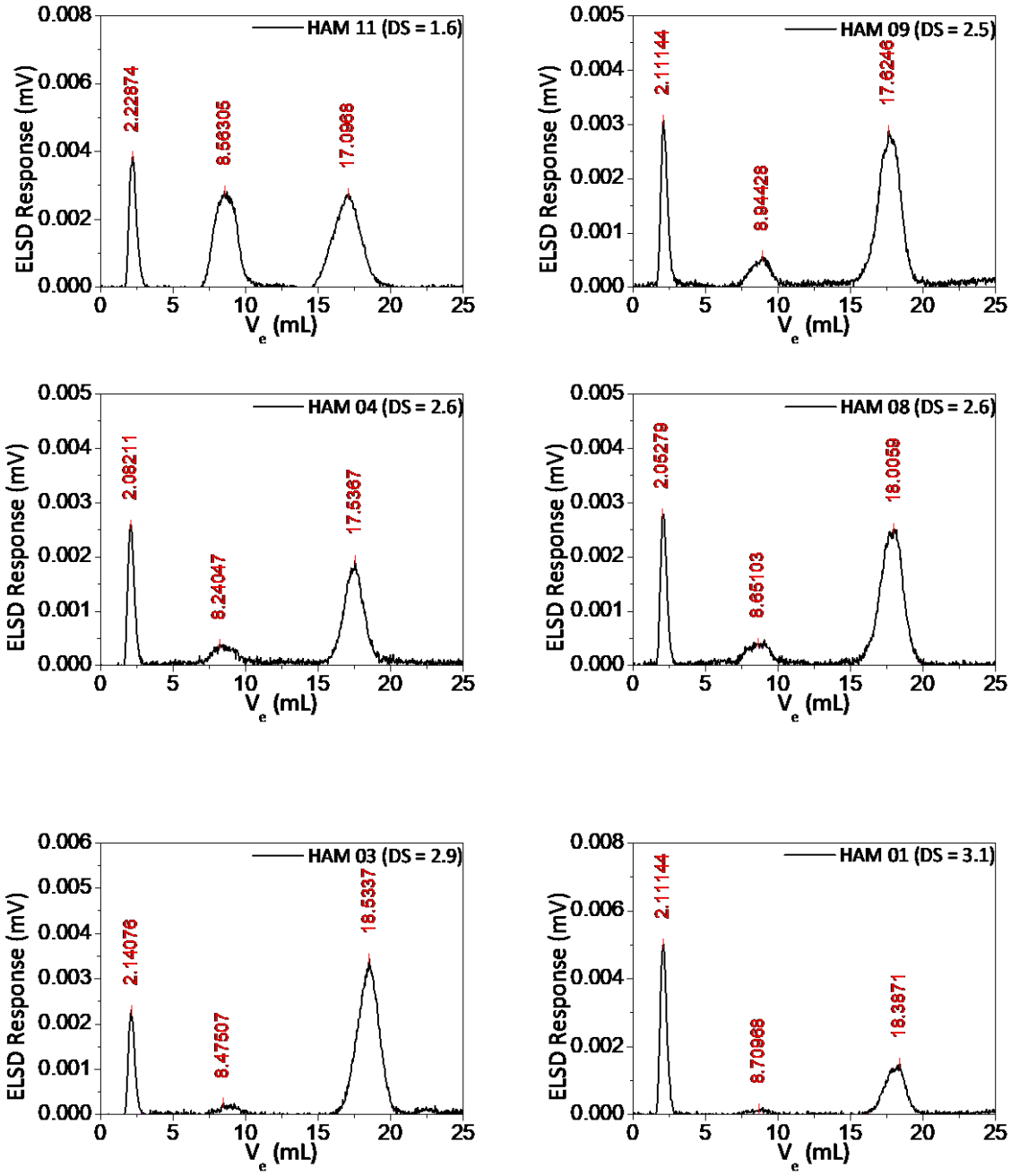

Figure 5.4 *Stepwise gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0.8 – 3.1). Stationary phase: Agilent Zorbax C8-RX (150 × 2.1 mm i.d.) with 5 µm particle size; mobile phase: H_2O-ACN (see* ***Table 5.2*** *for stepwise gradient profile). Sample solvent: ACN:H_2O (40:60 vol %) with 0.02 M ammonium acetate. All samples were stirred at 40 °C at 500 rpm for 20 hours in the absence of light.*

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In each case (**Figure 5.4**), the eluting peaks are broad which can be attributed to the fact that the samples are modified with the acrylate moiety to various extents, resulting in chemically heterogeneous samples. Therefore, a given modified HA sample not only contains polymer chains with high DS, but also polymer chains of low DS. For this reason, the determined DS is referred to as an average value. These results clearly show that the modification is a heterogeneous process that produces modified HA with a broad CCD.

The step-wise gradient method achieved baseline separation of various modified HA samples while still maintaining the ability to separate the unmodified HA from the modified HA. **Figure 5.5** shows the overlay of the gradient chromatograms of the HA samples varying in average DS values (DS = 0 – 3.1), while **Figure 5.6** is an enlarged overlay of a selected region of the gradient chromatograms presented in **Figure 5.5** to illustrate the separation of the modified HA according to average DS. Lastly, based on the elugrams shown in **Figure 5.7** it was concluded that the molar mass of the polymer does not influence the separation based on DS. HAM 04 (87000 $g.mol^{-1}$) and HAM 08 (96000 $g.mol^{-1}$), substituted to the same DS, had the same elution profile, regardless of molar mass.

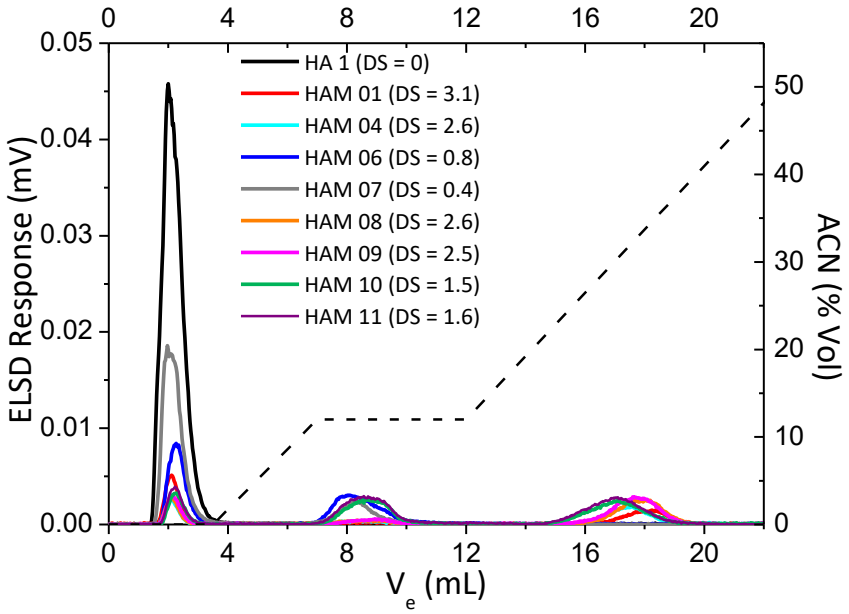

Figure 5.5 *Overlay of stepwise gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0 – 3.1).*

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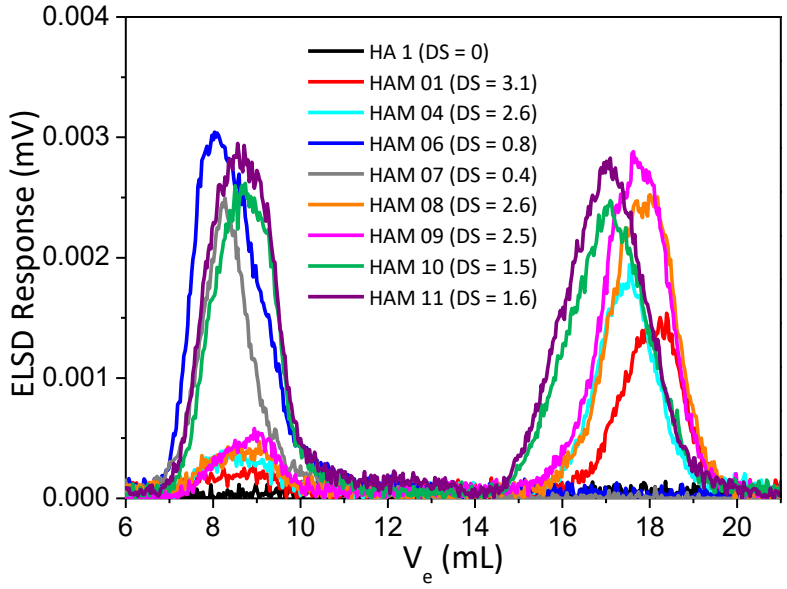

Figure 5.6 *Enlarged overlay of a selected regions of the gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0 – 3.1) presented in* ***Figure 5.5****.*

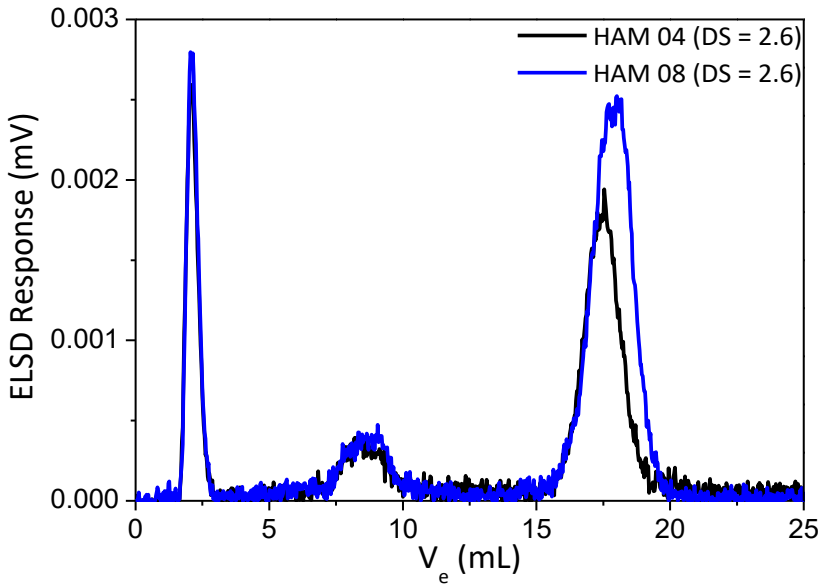

Figure 5.7 *Overlay of stepwise gradient HPLC chromatograms of HAM 04 (87000 g.mol^{-1}) and HAM 08 (96000 g.mol^{-1}), with the same average DS values (DS = 2.6).*

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5.3.2.1. Verification of the step-wise gradient method

Method validation is an essential part of HPLC method development. The efficiency and separation ability of the gradient HPLC method developed on the C-8 column was verified by injecting a blend containing HA samples that vary in average DS value. The two samples selected for the blend was HAM 06 (DS = 0.8) and HAM 01 (DS = 3.1). HAM 06 was selected due to the two distinct peaks present in the elution profile, the unmodified peak or breakthrough peak, and a lower DS modified HA component. HAM 01 had three peaks present in the elution profile when separated by the step-wise gradient. It contained the unmodified component or breakthrough peak and the lower and higher DS modified HA components. However, the lower DS component in the HAM 01 sample was negligible. The blend was successfully separated through the utilization of the step-wise gradient into its representative chemically distinct components (**Figure 5.8**).

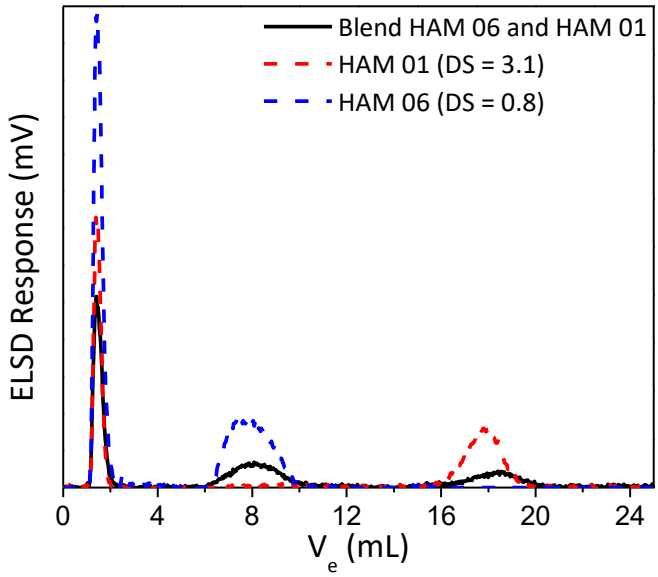

Figure 5.8 *Method verification by the separation of a blend of HAM 06 (DS 0.8) and HAM 01 (DS 3.1) with the step-wise gradient.*

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5.3.2.2. Analysis of the chemical composition by offline ^{1}H-NMR spectroscopy

To elucidate the chemical composition of the individual eluting peaks observed in the chromatograms in **Figure 5.4**, preparative fractionation and isolation of selected eluting peaks were conducted and the fractions analysed by ^{1}H NMR spectroscopy. The modified HA sample, HAM 10 (DS = 1.5) was chosen as the resolution of the baseline separation between the different chemically distinct components in this sample was the best. The individual fractions collected of HAM 10 (DS = 1.5), are shown in **Figure 5.9**.

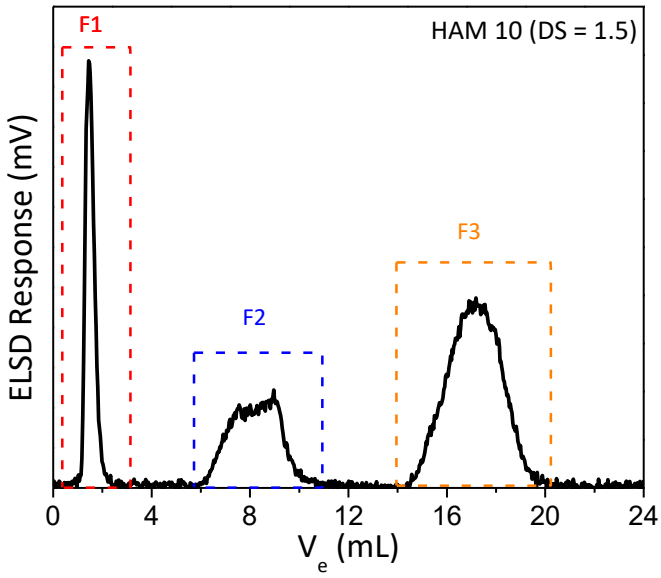

Figure 5.9 *Preparative fractionation and isolation of selected peaks of HAM 10 (DS = 1.5) to determine chemical composition. Sample concentration: 2.0 mg.mL^{-1}; Injection volume: 70 µL.*

Prior to sample fractionation and isolation, the influence of the experimental chromatographic parameters on the elution behaviour was investigated. The parameters investigated were the sample concentration and the injection volume (**Figure 5.10 (a)** and **Figure 5.10 (b),** respectively). The purpose of the investigation was to optimize these parameters to ensure that as much sample as possible can be injected and collected from a single run without compromising the selectivity and efficiency of the developed method.

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From the results, it was concluded that an increase in sample concentration in the given range had no significant effect on the elution behaviour of the polymer and the selectivity of the method. The dissolution of the sample above 2.0 mg.mL^{-1} became more unfavourable. However, the influence of injection volume on the first peak is evident at injection volumes of more than 50 µL. Nonetheless, it was decided to use a sample concentration of 2.0 mg.mL^{-1} and an injection volume of 70 µL to ensure that a sufficient amount of sample for ^{1}H NMR spectroscopy is obtained within a reasonable time.

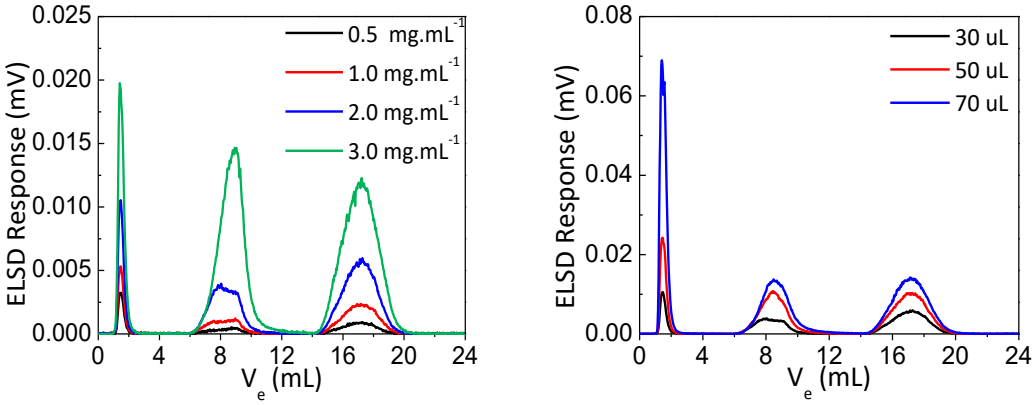

Figure 5.10 *HAM 10 (DS = 1.5) was (**a**) dissolved at various sample concentrations and (**b**) injected at various injection volumes (sample concentration of 2.0 mg.mL^{-1}) and then separated by the step-wise gradient method.*

The collected fractions of HAM 10 were dried under atmospheric conditions, in the absence of light, to minimise degradation.[8] The individual fractions were then dissolved in deuterium oxide (D_2O) and analysed by ^{1}H NMR spectroscopy to determine the average degree of substitution of each fraction. The individual ^{1}H NMR spectra for each of the fractions are shown in **Figure 5.11** (F1), **Figure 5.12** (F2) and **Figure 5.13** (F3). The average DS for each fraction was determined by using the following equation:

$$DS = \frac{\int (b+c,c')}{\int a}$$

Where *c, c'* and *b* are the protons attributed to the acrylate moiety and *a* are the methyl protons.

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From the ^{1}H NMR spectra of HAM 10 it was determined that fraction 1 had an average DS = 0.5, fraction 2 an average DS = 1.6 and fraction 3 an average DS = 2.2. The overall average DS of the collected fractions was 1.4, which is in line with the DS obtained for the bulk sample, 1.5.

The results obtained from ^{1}H NMR spectroscopy analysis confirmed that the second and third eluting peaks are attributed to HA with lower and higher average DS, respectively, while the first eluting peak could contain low DS HA polymer or a mixture of unmodified HA. Therefore, it is still uncertain if the first eluting peak is either attributed to (1) the unmodified HA, (2) the breakthrough effect or (3) a combination of both. Thus to obtain comprehensive information on the first peak, online 2D-LC is required (**Chapter 6**).

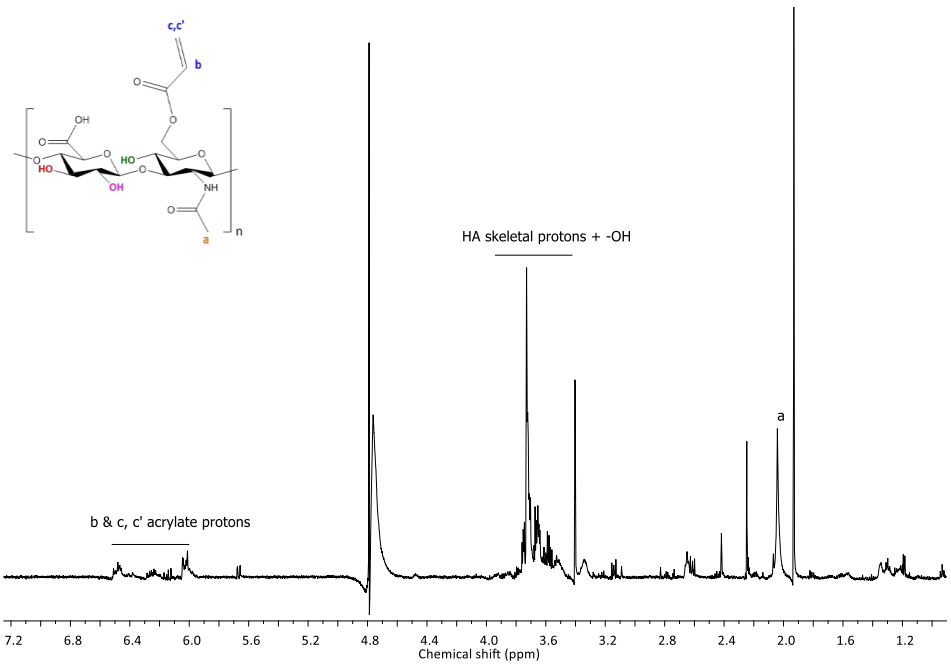

Figure 5.11 *^{1}H NMR spectrum of F1 (fraction 1) of HAM 10 (DS = 1.5) dissolved in D_2O. Fraction 1 was determined to have a DS = 0.5.*

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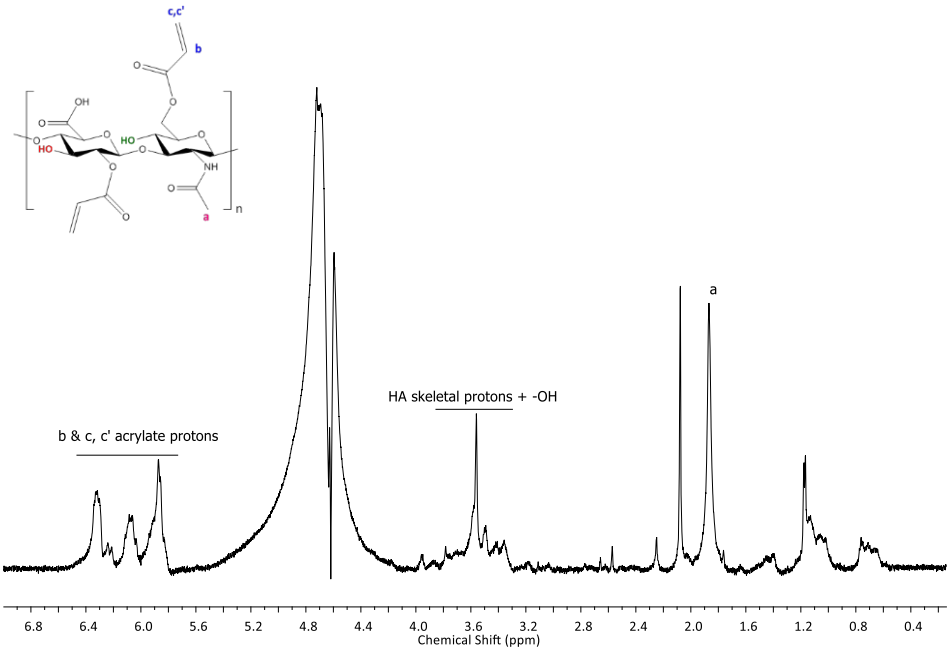

Figure 5.12 *^{1}H NMR spectrum of F2 (fraction 2) of HAM 10 (DS = 1.5) dissolved in D_2O. Fraction 2 was determined to have a DS = 1.6.*

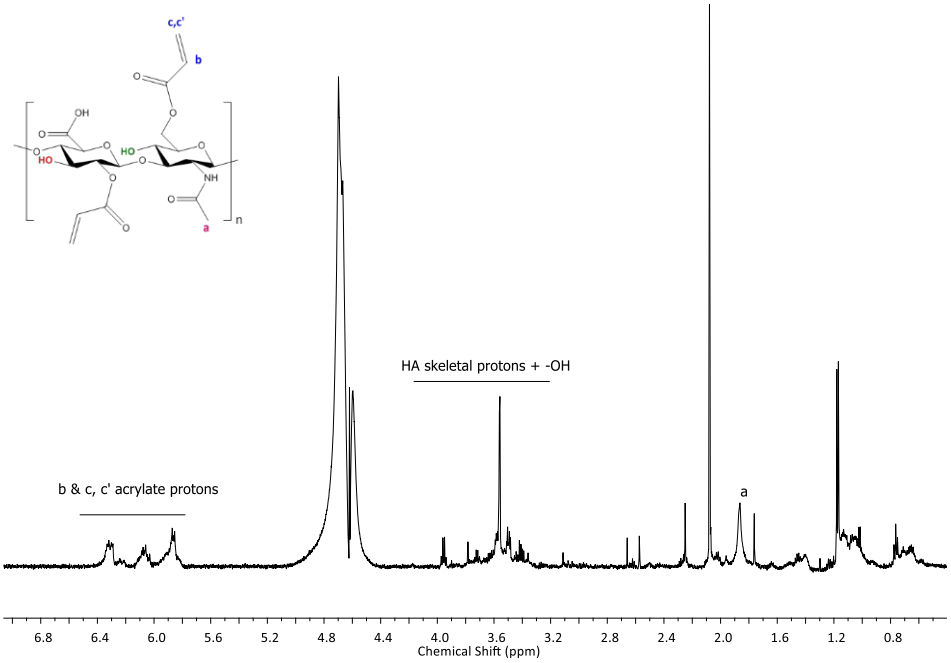

Figure 5.13 *^{1}H NMR spectrum of F3 (fraction 3) of HAM 10 (DS = 1.5) dissolved in D_2O. Fraction 3 was determined to have a DS = 2.2.*

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5.3.2.3. Efficiency and reproducibility of the step-wise gradient method.

In the development of a separation method, it is important to investigate the reproducibility of the developed method. The reproducibility of the method was studied by comparing the HPLC chromatograms of samples separately prepared and analysed by the step-wise gradient HPLC (see **Figure 5.14 (a)** and **Figure 5.14 (b)**). It was concluded that the samples behaved in the same manner to the solvent system and had a similar dissolution. **Table 5.3** and **Table 5.4** provide quantitative information of the peak height, elution volume and relative peak area. The data for HAM 09 sample correlated well, however for HAM 10, the peak area for peak 2 and peak 3 are different for each of the individually prepared samples. The difference can be attributed to the relative error that occurs during sample preparation and the use of a sensitive ELSD as a detector.

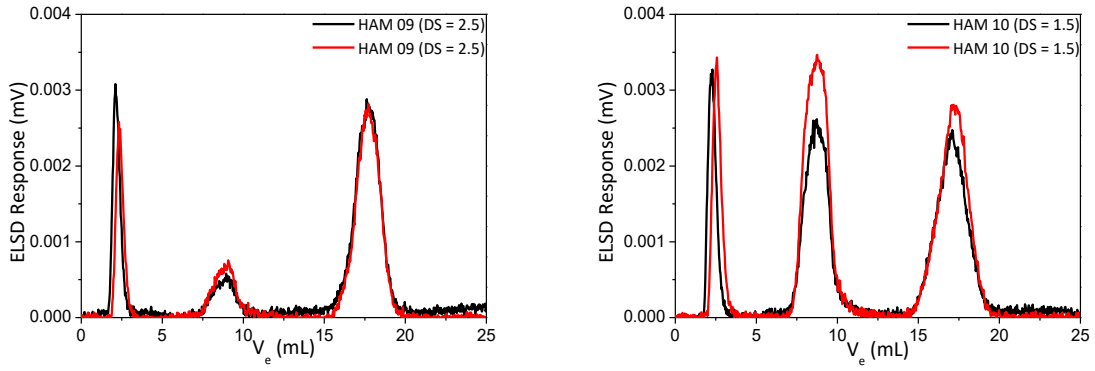

Figure 5.14 *Overlay of stepwise gradient HPLC chromatograms of two separately prepared samples of* ***(a)*** *HAM 09 (DS = 2.5) and* ***(b)*** *HAM 10 (DS = 1.5) to compare if the sample reacts in the same manner to the solvent system and is dissolved to the same extent.*

Table 5.3 Comparison of peak maxima, elution volume and relative peak area of HAM 09

HAM 09 (DS= 2.5)	**Peak 1**		**Peak 2**		**Peak 3**	
Chromatogram	Black	Red	Black	Red	Black	Red
Peak maxima (mL)	2.11	2.33	8.94	9.00	17.62	17.68
Relative peak area	0.0017	0.0014	0.0009	0.0011	0.0050	0.0054
V_e (mL)	1.5 – 3.5	1.6 – 3.5	7.2 – 10.4	7.2 – 10.4	15.2 – 19.9	15.2 – 19.8

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Table 5.4 Comparison of peak maxima, elution volume and relative peak area of HAM 10

HAM 10 (DS = 1.5)	**Peak 1**		**Peak 2**		**Peak 3**	
Chromatogram	Black	Red	Black	Red	Black	Red
Peak maxima (mL)	2.29	2.56	8.74	8.74	17.10	17.12
Relative peak area	0.0021	0.0022	0.0047	0.0062	0.0055	0.0061
V_e (mL)	1.6 – 3.5	1.8 – 3.6	7.0 – 11.4	7.0 – 11.0	14.4 – 19.5	14.6 – 19.5

5.3.2.4. Degradation study of modified hyaluronic acid

From previous degradation studies conducted by members of the Pasch group, it was discovered that the HA polymers were sensitive to degradation, especially when exposed to light and kept in solution.[4] From literature it is known that HA tends to degrade at elevated temperature and above and below pH 11 and pH 5, respectively.[8,13] Therefore, the stability of the HA samples in solution was investigated to make sure that the samples do not degrade during the analytical investigations. A freshly prepared sample was compared with a sample that was prepared 30 days before and stored in the absence of light. In **Figure 5.15** the overlays of recently prepared and 30 days previously prepared of the selected samples, HA 1 (DS = 0), HAM 06 (DS =0.8), HAM 09 (DS = 2.5) and HAM 10 (DS = 1.5), are presented. It was concluded that sample degradation due to solvent did occur to a small extent as the elution behaviour of the polymers were slightly changed, which means that the composition of the samples was altered while kept in solution. The degradation of the HA polymers in solution can be seen in each of the chromatograms as it is clearly illustrated by the change in peak intensities. The significant decrease in peak intensity observed in HAM 06 (peak 2) and in HAM 10 (peak 3) can effectively mean that less of that specific species were present after 30 days in the sample solution. The increase in peak intensity observed for HAM 10 (peak 2) suggested that more of that specific species are present in the sample solution after 30 days.

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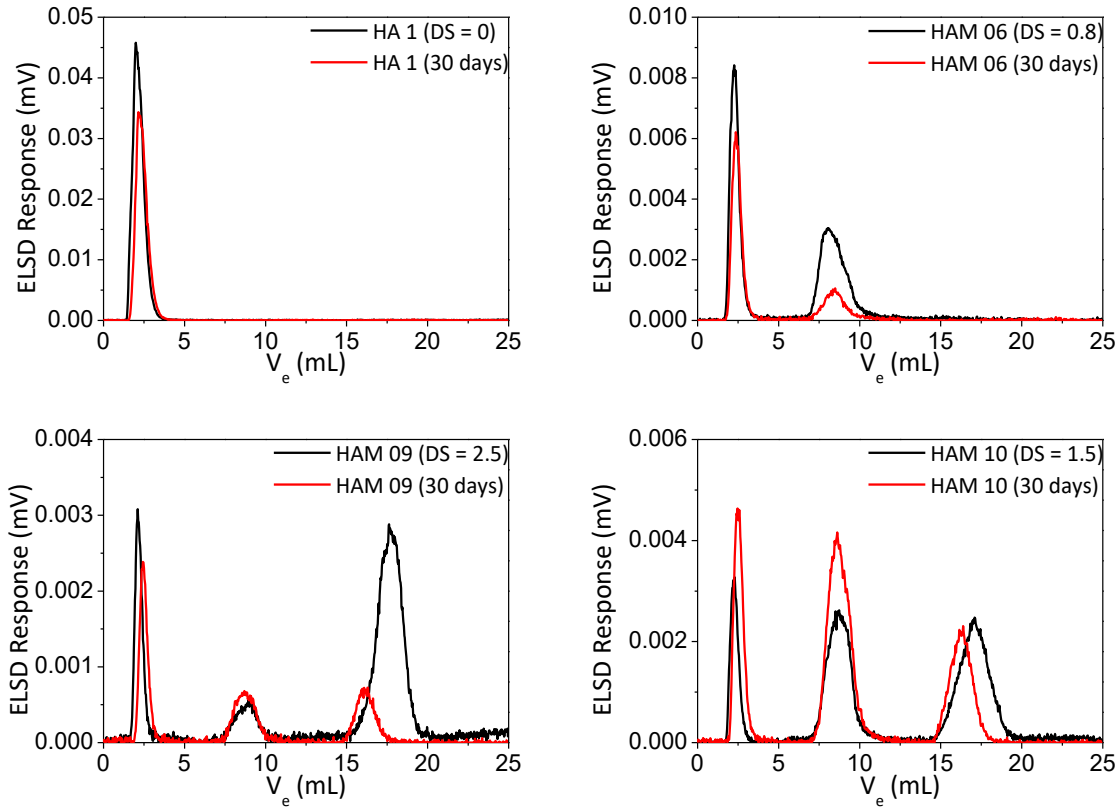

Figure 5.15 *Overlay of stepwise gradient HPLC chromatogram of HA samples recently prepared and HA samples prepared 30 days previously that were kept in the dark to study if the solvent system causes sample degradation*.

Furthermore, the stability of the HA samples over the course of the LC method development period was investigated. Similar to previous studies done in 2015 by members of the Pasch group,[4] it was observed that the composition of the samples had altered, as the solubility in ACN:H_2O (40:60 vol. %)/0.02 M ammonium acetate, DMSO:H_2O (60:40 vol. %), and DMSO:H_2O (60:40 vol. %)/0.05 M LiBr, had changed. Most of the samples were no longer soluble. For a few of the samples that were still soluble, the separation using the stepwise gradient was monitored. **Figure 5.16** shows the overlaid chromatograms for the individual samples. The first runs (**Run 1)** represents the separation achieved at the beginning (February/March 2017) of the study, **Run 2** and **Run 3**, are the intermediate runs, while **Run 4** represents the separation achieved at the end of the study (May 2017), with the stepwise gradient. It was found that there are significant changes in separation. The position of the first eluting peak changed for all samples. At the end of the study, it was observed that the first eluting peak eluted at the void volume of the column, hence no interaction with the

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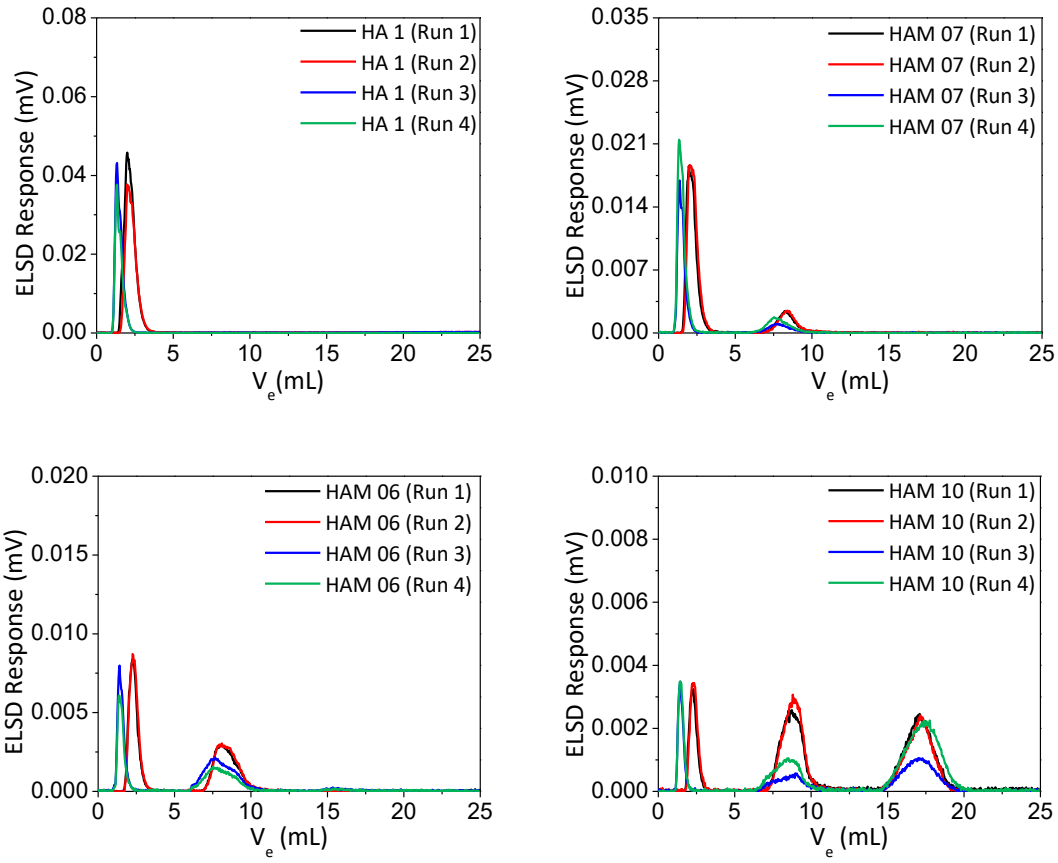
column occurred. Initially, the first eluting peak eluted after the void volume (1.1 mL), which meant that the analytes interacted with the column. It was noticed that for the majority of the HA polymer samples with high degrees of substitution ranging from DS = 2.5 to DS = 3.1 (HAM 09, HAM 04, HAM 08, HAM 03 and HAM 01), that over the course of the study the intensity of the second peak decreased, and subsequently was not detected. The last eluting peak of the high DS samples was lower in intensity and the peak shape changed as it was broader compared to the initial analysis of the samples. Nonetheless, for all the samples the elution of the peak still started at the same elution volume. For HAM 10 (DS = 1.5) and HAM 11 (DS = 1.6), the second eluting peak was still present; however, the peak intensity, peak shape and elution volume had changed. For both HAM 10 and HAM 11, no changes in peak intensity and elution volume were observed for the last eluting peak at the end of the study (Run 4), but the peak was broader. For the low degree of substitution (HAM 06 and HAM 07) HA polymers, the peak intensity of the second eluting peak decreased, along with a shift in elution volume and change in peak shape. It was thus evident that the composition of the samples changed during the course of the project. The decrease in intensities could also be attributed to the decreased solubility of the samples.



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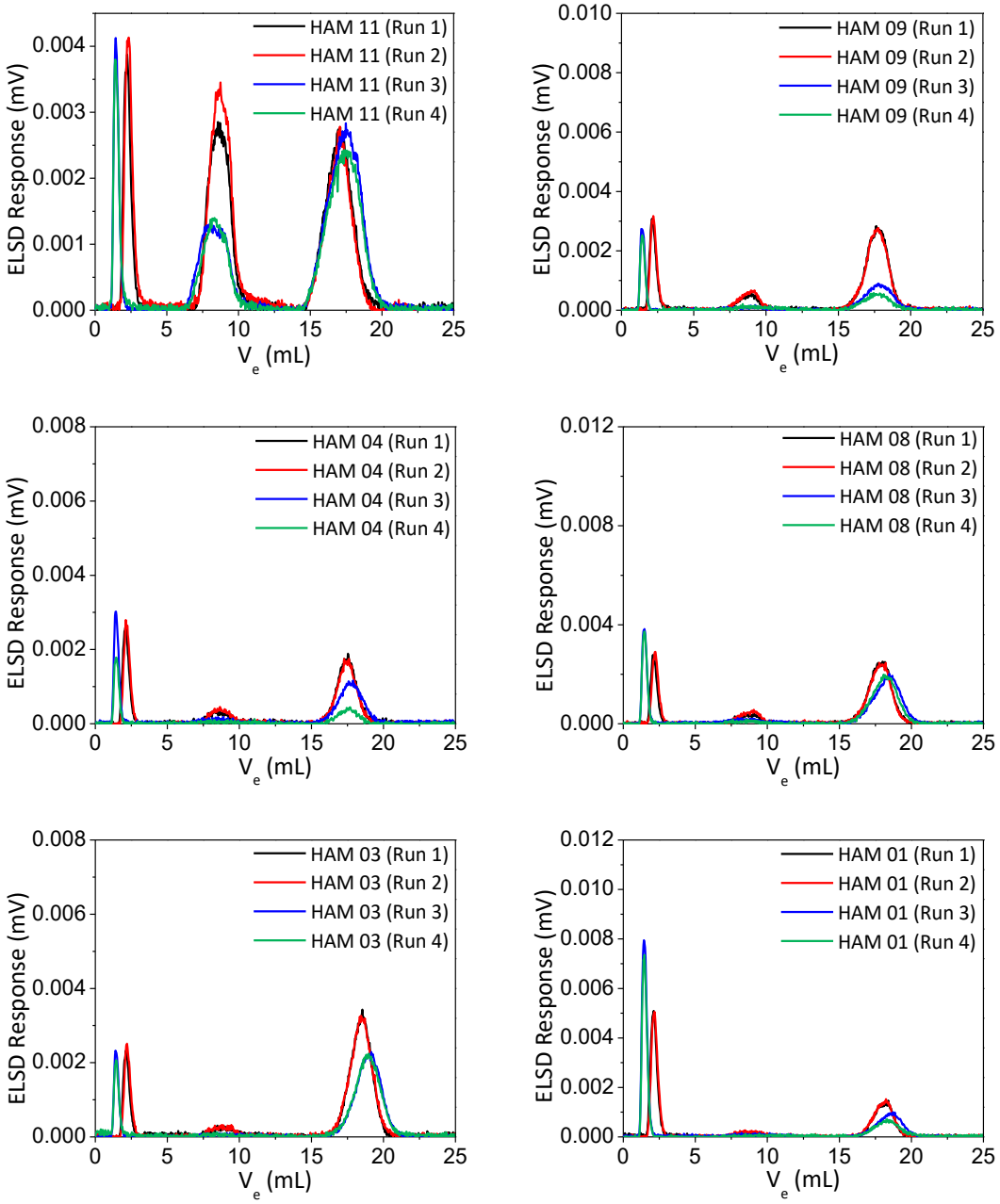

Figure 5.16 *Overlay of chromatograms of HA 1 to HAM 11 (DS = 0 – 3.1) analysed during the course of the study, where Run 1 (**black**) is the initial analysis and Run 4 (**green**) is the most recent analysis of the sample.*

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The next step in the study was to investigate the effect of temperature on the separation of the HA samples (see **Figure 5.17**). The aim was to establish if the separation would be improved at elevated temperatures. Earlier temperature studies done of the modified HA samples showed that the polymers degrade above the temperature of 40 °C.[2] Therefore the analysis temperatures tested were 30 °C, 35 °C and 40 °C. All samples were dissolved at a temperature of 40 °C, as it was previously established that the solubility of the HA polymers improved at a higher temperature (see Chapter 3). From the results of HAM 09 and HAM 10, it was clear that an increase in temperature improved the selectivity of the samples, as the initial peak eluted after the void volume of the column. For both 30 °C and 35 °C the first peak eluted before the void volume of the column, indicating that the analytes did not interact with the column. The intensity of the second peak was enhanced with less peak fronting being observed at 40 °C. It would thus appear that the increase in temperature assisted in either reducing the aggregate formation and/or improved the solubility of the samples. The results obtained correlated well with the solubility studies conducted (see Chapter 3) and with the previous temperature study conducted on the same samples dissolved in a different solvent system.[4] It was therefore concluded, that the most suitable temperature for all LC experiments would be 40 °C.

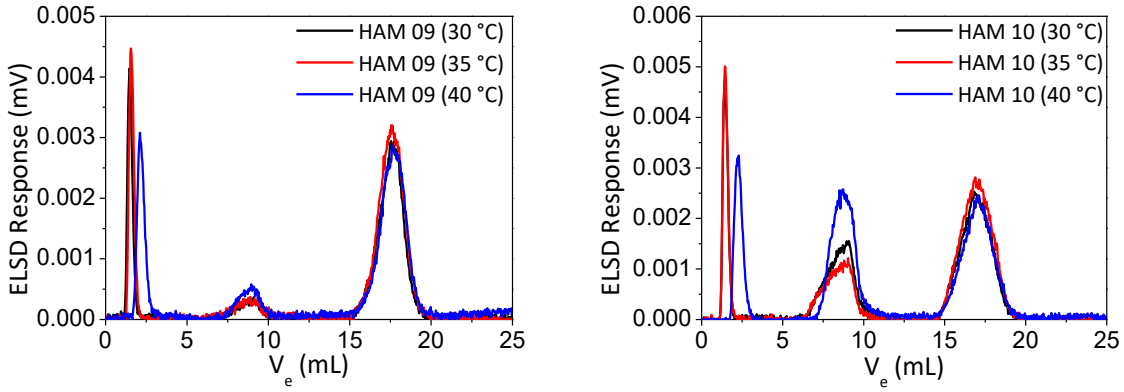

Figure 5.17 *Overlay of stepwise gradient HPLC chromatogram of* ***(a)*** *HAM 09 (DS = 2.5) and* ***(b)*** *HAM 10 (DS = 1.5) obtained at various temperatures.*

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5.4. Conclusion

A reliable and robust HPLC method was developed for the separation of the unmodified HA and modified HA in the range of DS = 0 to 3.1. The developed method was able to separate unmodified HA from modified HA, while separating the modified HA according to the DS. More interestingly, it was found that the separation and selectivity of the modified HA improved with the addition of a volatile salt. The ability of the developed method to separate the HA polymers according to different degrees of substitution was validated by the analysis of the various eluting components by ^{1}H NMR spectroscopy. ^{1}H NMR confirmed that the separation on the C-8 column was according to increased hydrophobicity, in other words, HA polymers with higher degrees of substitution were more retained.

5.5. References

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Chapter 6

Two-dimensional Liquid Chromatography Method Development

This section will focus on the optimization of reversed phase liquid chromatography for the two-dimensional liquid chromatography (2D-LC) analysis. Also included in this section is the optimization of the 2D-LC method followed by the discussion of the 2D-LC results obtained.

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6.1. Introduction

Modified HA is heterogeneous in terms of both chemical composition and molar mass distribution.[1-4] Modified HA samples inherently contain a complex chemical architecture, as substitution with an acrylate moiety occurs randomly and not homogeneously along the backbone of the polymer.[2] In order to accomplish a full and comprehensive characterization of hyaluronic acid and its derivatives, advanced chromatographic techniques are required to establish reliable structure-property relationships of the polymer. The correlation between the DS and the molar mass of the different HA samples cannot simply be done using one-dimensional LC. Interaction chromatography predominantly separates according to chemical composition, and as a result, molar mass information is lost.[5] At the same time, SEC separates according to the hydrodynamic volume, thus molecules of different chemical composition having the same hydrodynamic volume will co-elute.[5-7] Using multi-dimensional LC, separation according to two different parameters e.g. chemical composition and molar mass can be obtained simultaneously.[8,9] On-line multi-dimensional analysis eliminates tedious fraction collections, minimizes sample degradation and improves the structural characterization of complex polymers. In this work, separation according to the degree of substitution was achieved in the first dimension, and the fractions obtained were transferred to the second column for separation according to hydrodynamic volume. The collected fractions consist of polymer chains that have approximately the same DS, regardless of the position of the substitution along the polymer backbone. By calibrating the second dimension, the molar mass distributions of these polymer fractions were obtained.[10] A universal ELSD was chosen for 2D-LC detection due to its high sensitivity and versatility. The developed individual one-dimensional LC methods had thus to be compatible with each other and also with the ELSD.

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6.2. Experimental and Instrumentation

6.2.1. Solvents and Chemicals

Acetonitrile (ACN) (HPLC grade, Sigma-Aldrich), water (H_2O) (Millipore from laboratory H_2O filtration system) and ammonium acetate ($NH_4CH_3CO_2$). The solvents and salt were used as received.

6.2.2. 2D-LC with ELSD for multi-dimensional analysis.

Two-dimensional liquid chromatography (2D-LC) analysis was performed on an Agilent 1200 series instrument equipped with a vacuum degasser, a quaternary pump, an isocratic pump, an autosampler, a column oven, and an Agilent 1260 infinity evaporative light scattering detector (ELSD). The column oven for both the first dimension and second dimension column was kept at 40 °C. An Agilent Zorbax C8-RX column (150 × 2.1 mm i.d.) with 5 µm average particle size was used in the first dimension. An injection volume of 30 µL of a 2.0 $mg{\cdot}mL^{-1}$ sample was injected onto the column at a flow rate of 0.053 $mL{\cdot}min^{-1}$. The mobile phase for step-wise gradient HPLC comprised of 100 vol. % H_2O to ACN:H_2O 50:50 vol. %. The subsequent fractions collected from the first dimension separation were transferred with the aid of an electronically controlled Valco 8-port switching valve consisting of two 200 µL storage loops. The fractions were subjected to further analysis in the second dimension. The second dimension consisted of a PSS-Suprema High-speed (20 mm × 50 mm, medium linear) column. An isocratic mobile phase composition was used for separation in the second dimension at a flow rate of 4.0 $mL{\cdot}min^{-1}$. The mobile phase comprised of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate. The ELSD was set to an operating temperature of 100°C and the sensitivity (gain) of the detector to 9 mV. The system was calibrated using narrow poly(ethylene oxide) standards ranging from 900 to 1,000,000 $g{\cdot}mol^{-1}$. Data acquisition and calibration were done with WinGPC Unity software (version 7, PSS Polymer Standards Service GmbH, Mainz, Germany). All data processing was done with OriginPro 8.0.

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6.2.3. Sample preparation

Samples with a concentration of 2.0 mg·mL^{-1} were prepared by dissolving 4.0 mg of sample in 2.0 mL of ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate solvent. The dissolution occurred for 20 hours at 40 °C at 500 rpm stirring rate, in the absence of light and filtered through a 0.45 µm regenerated cellulose filter before analysis.

6.3. Optimization of stepwise gradient method for two-dimensional liquid chromatography.

The online hyphenation of HPLC and SEC for multi-dimensional analysis can be a time-consuming process as two separate one-dimensional LC methods are combined. In 2D-LC the experimental parameters of the first dimension are dependent on the experimental parameters of the second dimension.[8,11] For example, the flow rate used for the second dimension determines the flow rate for the first dimension as the fractionation and sample transfer time are subsequently dependent on the flow rate. The analysis of the fraction transferred to the second dimension must be completed before another fraction can be loaded onto the second dimension column and analysed. Thus, the optimization of the first dimension method, regarding the analysis time, was required to achieve separation in the least possible time. The developed stepwise gradient (see Chapter 4) was optimized as tabulated and in **Table 6.1** and **Figure 6.1**, respectively.

Table 6.1 Optimized stepwise gradient profile

V_e (mL)	ACN (vol. %)	H_2O (vol. %)
0	0	100
3.5	0	100
4	12	88
7.5	12	88
12.5	40	60
14	40	60
15	0	100
22.5	0	100

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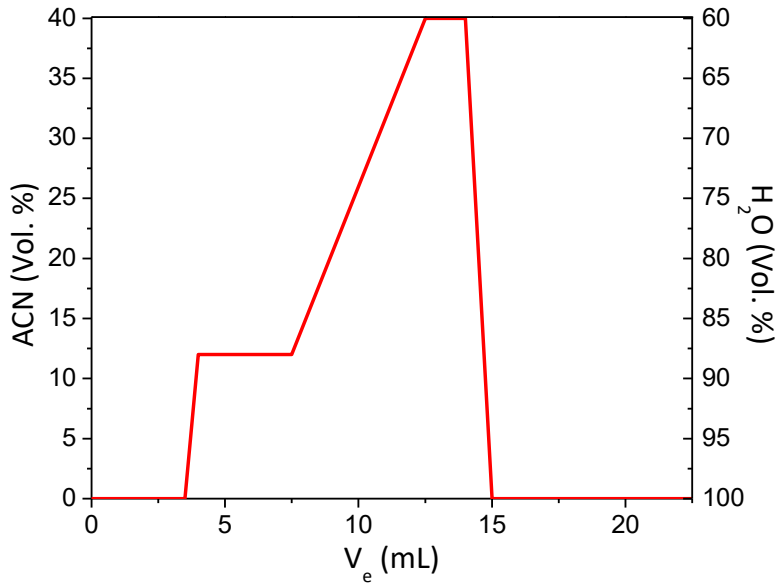

Figure 6.1 *Optimized stepwise gradient used for the first dimension of the two-dimensional separation of the modified and unmodified HA with varying degrees of substitution (DS = 0 - 3.1). Stationary phase: Agilent Zorbax C8-RX (150 × 2.1 mm i.d.) with 5 µm particle size; mobile phase: H_2O-ACN (see* ***Table 6.1*** *for stepwise gradient profile).*

The analysis time in the first dimension was reduced from 70 min to 40 minutes. The individual chromatograms of representative HA samples (HA 1 (DS = 0), HAM 04 (DS = 2.6), HAM 06 (DS = 0.8), HAM 10 (DS = 1.5) and HAM 11 (DS = 1.6)) are shown in **Figure 6.2.** The selectivity of each of the separations remained constant as three distinct peaks were observed for the higher average DS modified samples, while two peaks were observed for the lower average DS modified samples.

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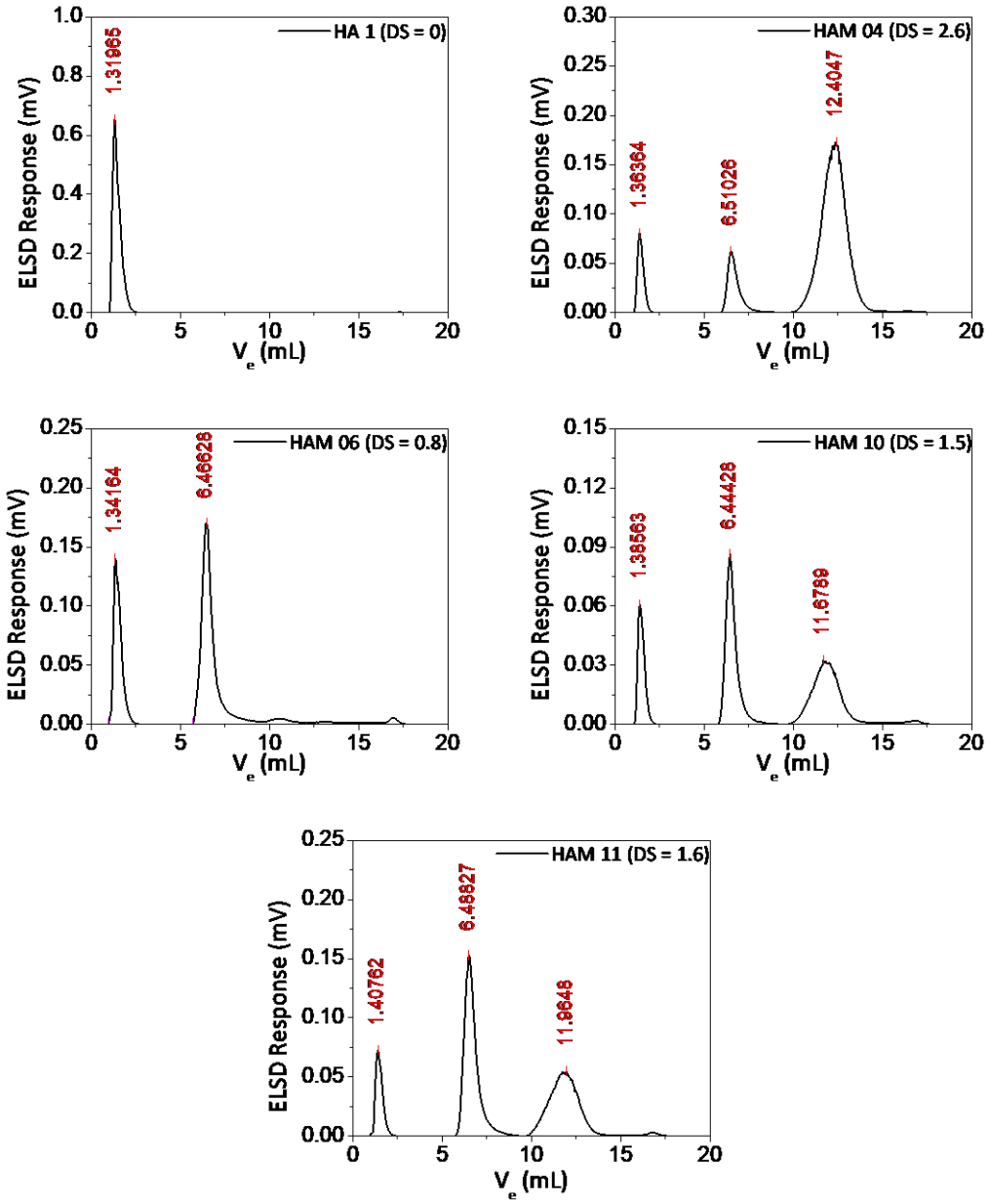

Figure 6.2 *Optimized step-wise gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0.8 – 2.6).*

Baseline separation and separation according to DS were still achieved with the optimized stepwise gradient as illustrated in **Figure 6.3.** An enlarged overlay of a selected region of the elugrams of HA samples with varying DS values (DS = 0 – 2.6) is presented in **Figure 6.4**. From the elugrams, it was clear that with the optimization of the new step-wise gradient, the resolution was slightly lower compared to **Figure 5.4** (see **Chapter 5**).

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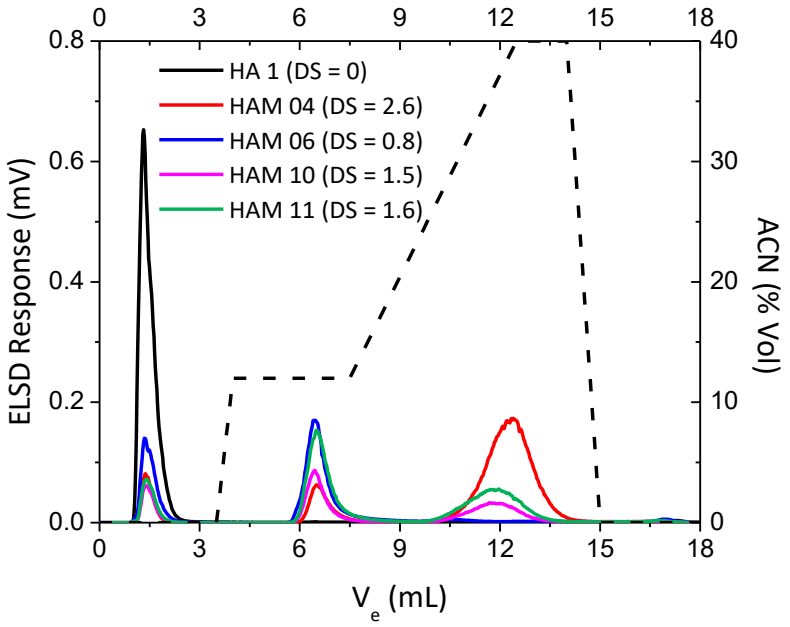

Figure 6.3 *Overlay of the optimized step-wise gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0 – 2.6).*

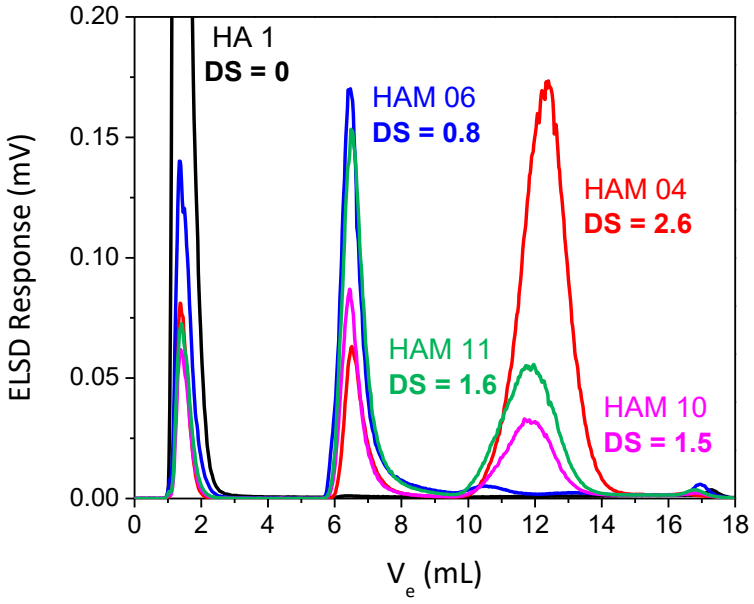

Figure 6.4 *Enlarged overlay of a selected region of the gradient HPLC chromatograms of HA samples varying in average DS values (DS = 0 – 2.6) presented in* ***Figure 6.3****, to illustrate the baseline separation achieved.*

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6.4. Optimization of the chromatographic parameters for 2D-LC.

During the process of fractionation (collection and transferring to the 2nd dimension), the sample is continuously diluted.[12] In order to conduct a 2D-LC analysis on a sample, the chromatographic parameters require optimization to ensure that an adequate detectable amount reaches the detector (ELSD) after being subjected to both the first and second dimensions.[12] The chromatographic parameters, sample concentration and injection volume were investigated. The results are shown in **Figure 6.5**. It is shown that with an increase in sample concentration, the baseline separation was significantly influenced, as the resolution was lower. The increased injection volume had no significant effect on the elution behaviour of the samples (see **Figure 6.6**). It was concluded that suitable experimental conditions for 2D-LC were a sample concentration of 2.0 mg·mL^{-1} with an injection volume of 30 µL.

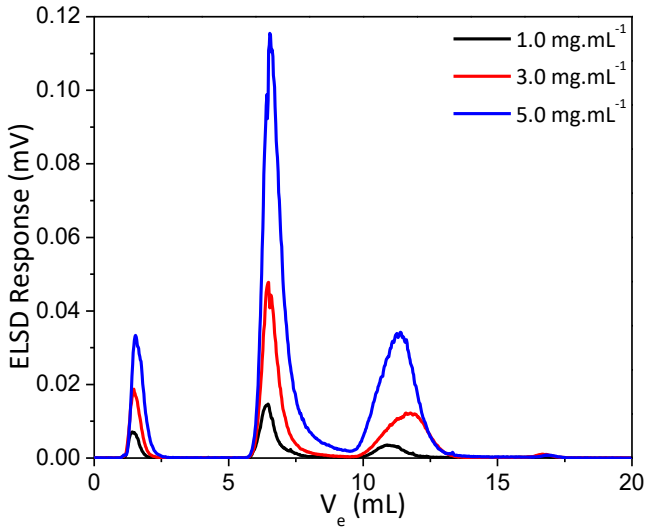

Figure 6.5 *Elugrams of HAM 10 (DS = 1.5) which was dissolved at various sample concentrations, injected and separated using the optimized step-wise gradient method to determine the optimal sample concentration for 2D-LC.*

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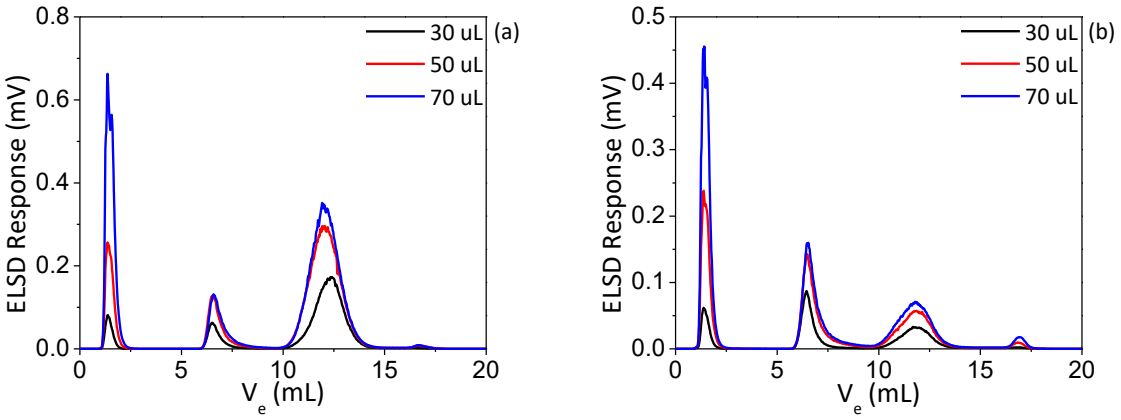

Figure 6.6 *Elugrams of* ***(a)*** *HAM 04 (DS = 2.6) and* ***(b)*** *HAM 10 (DS = 1.5) which were dissolved at a sample concentration of 2.0 mg.mL^{-1} and separated by the optimized step-wise gradient method. The injection volume was varied to determine the optimal injection volume for 2D-LC.*

6.5. Results and discussion

Separation in the first dimension was according to DS, while in the second dimension, the separation was achieved according to hydrodynamic volume, which is related to the size of the polymer in solution. For unmodified HA, only one component was observed in the 2D-LC experiments. It is clear from **Figure 6.7**, which illustrates the individual chromatograms of each of the unmodified HA fractions, that the sample is heterogeneous with regards to molar mass as bimodality was observed for the majority of the fractions. The HA sample was shown to be highly polydisperse as component 1 eluted from 4.7 – 7 mL, which can be seen in the contour plot (see **Figure 6.9**).

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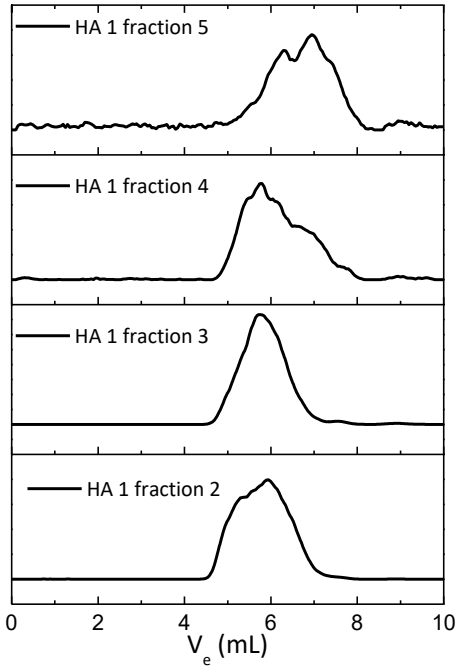

Figure 6.7 *SEC chromatograms obtained from the second dimension separation of the individual fractions of the first HPLC eluting peak of the unmodified HA.*

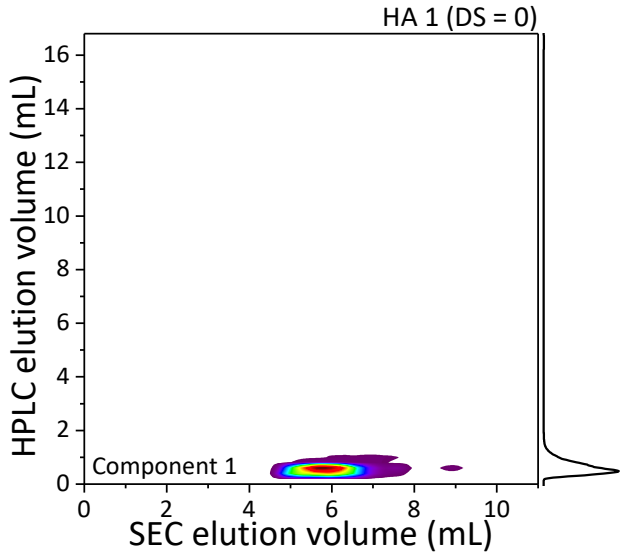

Figure 6.8 *2D-LC contour plot of unmodified HA 1 (DS = 0).*

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Figure 6.9 presents the SEC chromatograms of each of the individual fractions collected for the first HPLC eluting peak and the second HPLC eluting peak of samples HAM 04 (DS = 2.6) and HAM 10 (DS = 1.5), respectively. The contour plots for the separations achieved by two-dimensional analysis of the modified HA are shown in **Figure 6.10**. It was evident from the results that the individual fractions collected from the first dimension had broad molar mass distributions. This can be attributed to the heterogeneity of the samples with regard to molar mass or the presence of high molar mass aggregates. The formation of aggregates would result in larger hydrodynamic volumes and hence higher calculated molar masses.

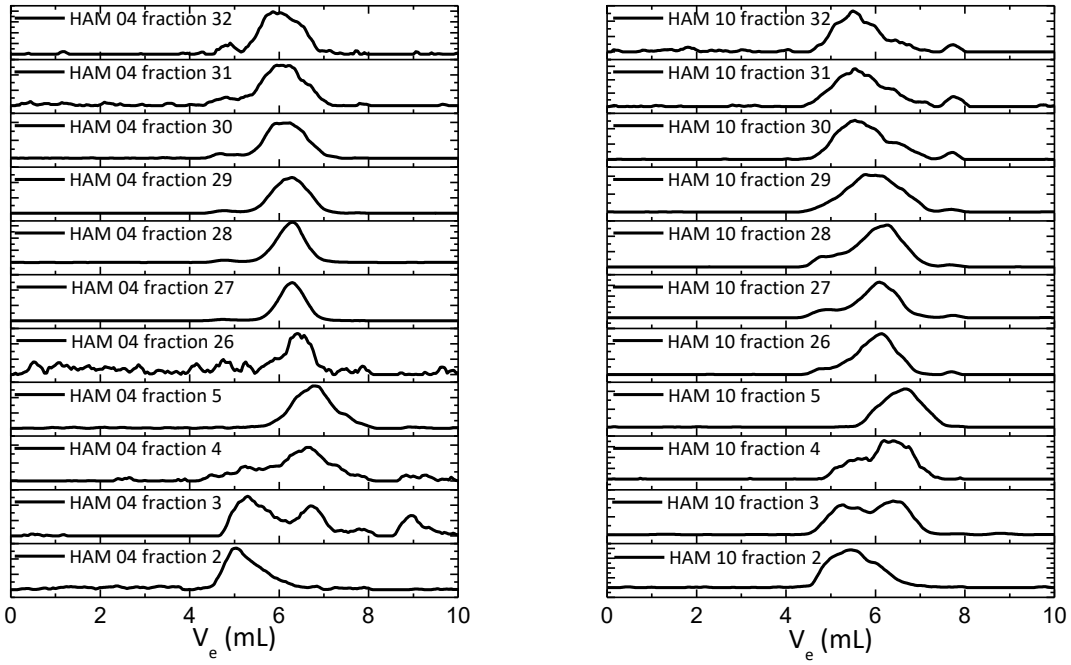

Figure 6.9 *SEC chromatograms obtained from the second dimension separation of the individual fractions of the first (Fraction 2 – 5) and second (Fraction 26 – 32) HPLC eluting peak for HAM 04 and HAM 10.*

As expected, once modified, HA consisted of several peaks, as observed in the first dimension, due to the different DS as determined by NMR spectroscopy. It was clear from the contour plots that a HA sample of low DS, had the highest concentration of molecules at the low DS (peak 2 in the first dimension). A HA sample of higher DS, the concentration profile was shifted and the majority of the molecules was at the higher DS peak (the last eluting peak in the first dimension). The first eluting peak of the HPLC dimension was shown

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to consist of at least two components of significantly different molar masses, unmodified HA and unretained modified HA, in the SEC second dimension. Given that HA 1 was the parent polymer to HAM 04 (DS = 2.6) and HAM 10 (DS = 1.5), it was assumed that the first component could be attributed to unmodified HA, as Component 1 for HAM 10 also eluted from 4.7 – 7 mL. Component 1 of HAM 04 eluted from 4.7 – 6 mL, however, this still corresponds to the molar mass range of the unmodified HA. HAM 04 was modified to a higher degree of substitution compared to HAM 10, and subsequently less unmodified polymers are present. Component 1 of HAM 10 had two peak maxima, thus, the molar mass was bimodal, which correlates to the elution profile observed for the individual parent HA 1 fractions.

Component 2 for HAM 04 and HAM 10 were, therefore, attributed to unretained modified HA of a significantly lower molar mass. However, it could also be assumed to be the breakthrough peak as it occurs at the void volume of the column.[13] But for the breakthrough peak to occur it would be assumed to have molar masses distributions similar to the other components. So, it is more likely that this is not the breakthrough effect but unretained low molar mass species. For clarification, a long polymer chain of higher molar mass and a short polymer chain of lower molar mass can be modified to the same degree per monomer unit i.e. have the same average DS value. This effectively means that the longer polymer chain contains more interaction sites that can interact with the column, than the short polymer chain.

Based on the ^{1}H NMR data obtained for each of the peaks that eluted in the first dimension (see Section 5.3.2.2), it was determined that the first peak had an average DS = 0.5. Thus the assumption for all modified HA samples would be that Component 1 is the unmodified HA and Component 2 is an unretained lower molar mass polymer.

Even though HAM 06 (DS = 0.8) and HAM 11 (DS = 1.6) had different parent polymers to that of HAM 04 and HAM 10, comparable elution profiles were observed for the first HPLC peak as it also consisted of more than one component. For HAM 06, component 1 had two peak maxima, similar to HAM 10, indicating the bimodality of the parent unmodified HA sample. The contour plot of HAM 11 showed that the first HPLC eluting peak was composed

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of three components. Component 1 was of higher molar mass, corresponding to the heterogeneous unmodified HA, while Component 2 and Component 3 were of lower molar mass and thus the unretained polymer.

For the second HPLC peak of the modified samples, two maxima were observed. This indicated that similar average DS species may consist of different molar masses. This can also be an indication of the different heterogeneities (1^{st} and 2^{nd} order heterogeneities) within the sample. The bimodality of each of the fractions that made up the second HPLC eluting peak was clear in HAM 06 (Component 3) and HAM 11 (Component 4), as two distinct maxima were observed. In the case of HAM 04 and HAM 10, the one maximum was of low intensity, and occurred as a shoulder present in the chromatogram of each of the individual fractions of both HAM 04 and HAM 10. The maximum of higher intensity was attributed to the peak (see **Figure 6.9**).

The third HPLC peak visible in samples HAM 04, HAM 10 and HAM 11, only contained one observable maximum, which indicates that the higher average DS have a unimodal broad molar mass distribution. However, HAM 06, which was of low DS, appeared to have a peak in the higher DS range. This was not visible from the one-dimensional analysis. Only after the sample had been analysed by multi-dimensional analysis did this become apparent. It shows again the complexity of the HA polymers and that it is imperative to analyse these polymers by two-dimensional LC for comprehensive analysis.

A minor component which eluted at 16 mL in the HPLC dimension and at 5 mL in the SEC dimension was observed in all modified HA samples when analysed by 2D-LC. This component was very prominent in HAM 10 (Component 5), compared to the other modified HA samples. The intensity of the late eluting component was very low for HAM 04, HAM 06 and HAM 11. The late eluting component is unknown; it could be a highly retained polymer (polymer of very high DS) as it eluted close to the end of the gradient or simply be a system peak. This peak was not observed in the analysed samples using the one-dimensional LC stepwise gradient (Section 5.3.2).

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It is evident from the multi-dimensional LC results that the substitution of the hydroxyl groups of the unmodified HA with the acrylate moiety was not homogeneous. From literature it is known that the primary OH is more reactive than the three secondary OH groups present in the monomer unit, as it is less electron-dense and sterically hindered.[4] The result is that there is not only a difference in the degree of substitution per monomer unit, but along the HA polymer backbone as well. The degree of substitution can also vary between the polymer chains of different length and size. The complexity and sample heterogeneity was illustrated in the contour plots of the modified HA, as it was apparent that the samples had a distribution with regard to DS, and within a specific DS, a bimodal molar mass distribution was also observed.

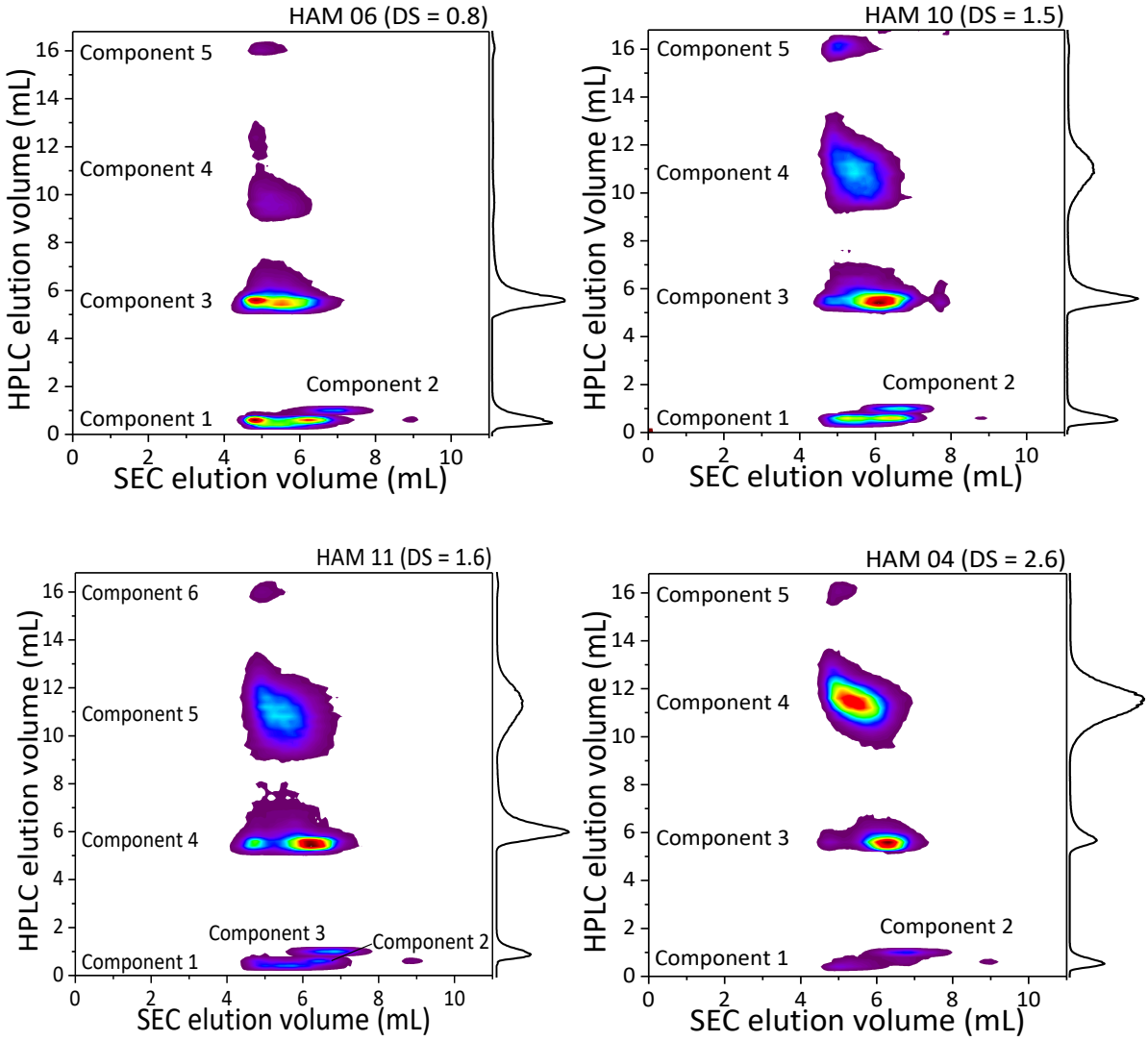

Figure 6.10 *2D-LC contour plot of modified HA in order of increasing DS, HAM 06 (DS = 0.), HAM 10 (DS = 1.5), HAM 11 (DS = 1.6) and HAM 04 (DS = 2.6).*

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6.6. Calibration of the second dimension

The calibration of the second dimension was achieved by injecting PEO calibration standards under the same 2D-LC experimental conditions determined for the HA samples. In the first dimension step-wise gradient HPLC is applied to achieve separation, thus the mobile phase composition was continuously changing. This meant that the fractions collected from the first dimension had varying solvent compositions. To investigate the effect of the solvent composition on the elution behaviour and molar mass determination, the PEO standards were injected at varying mobile phase compositions of ACN:H_2O as indicated in **Figure 6.11**. It was found that a change in solvent composition had no significant effect on the elution behaviour of the different PEO standards. The calibration curves remained the same. The calibration curve used for the molar mass determination of the HA samples eluting in the second dimension is shown in **Figure 6.12**.

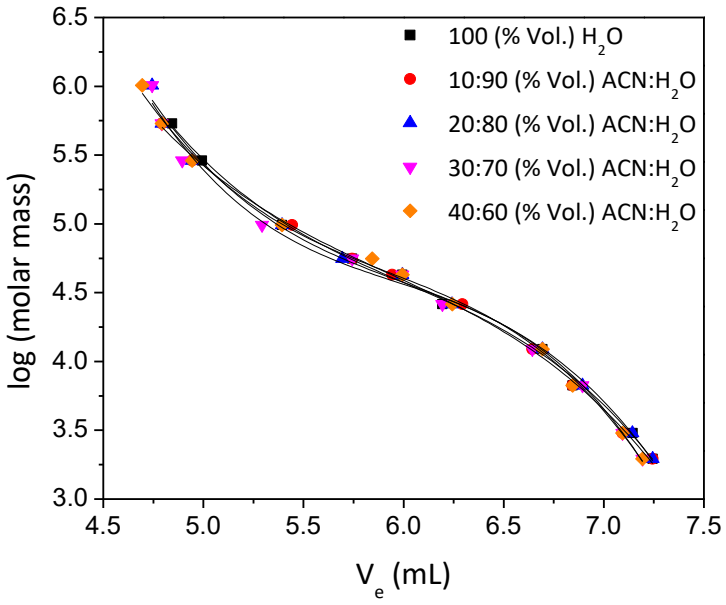

Figure 6.11 *Calibration curves obtained with PEO standards that were separated at varying mobile phase compositions. Sample solvent: ACN:H_2O (40:60 vol. %) with 0.02 M ammonium acetate.*

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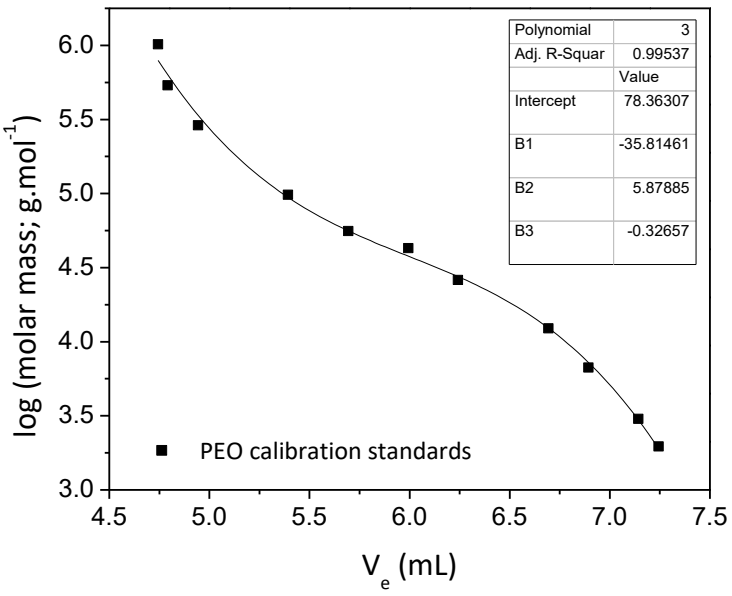

Figure 6.12 *Calibration curve obtained with PEO standards used for the molar mass determination of the HA samples. Sample solvent: ACN:H_2O (40:60, vol. %) with 0.02 M ammonium acetate.*

The molar mass and molar mass distributions of the selected HA samples (HA 1 (DS = 0), HAM 04 (DS = 2.6), HAM 06 (DS = 0.8), HAM 10 (DS = 1.5) and HAM 11 (DS = 1.6)) determined using a PEO calibration, are shown in **Figures 6.13** and **6.14**.

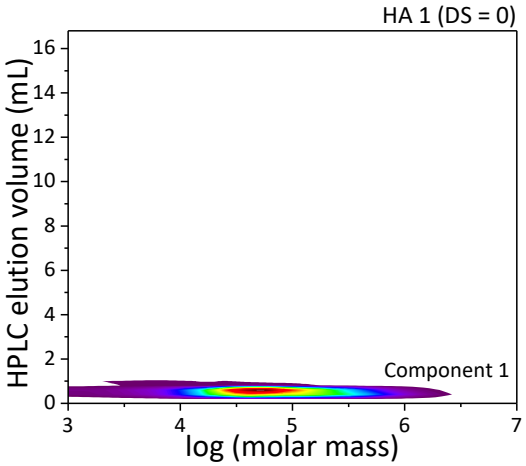

Figure 6.13 2D-LC contour plot of HA 1 (DS = 0). The second dimension, SEC, calibrated with PEO standards.

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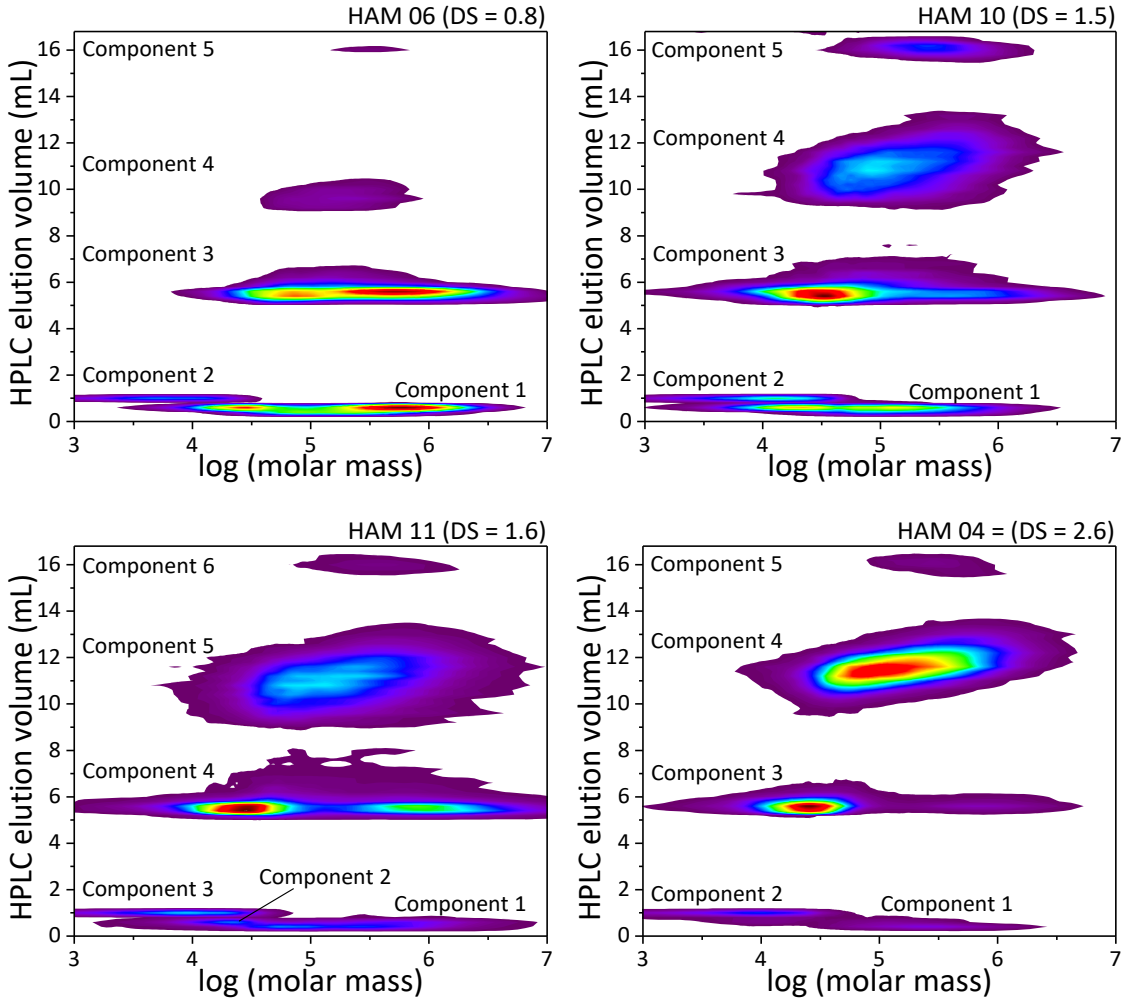

Figure 6.14 *2D-LC contour plot of modified HA in order of increasing DS, HAM 06 (DS = 0.), HAM 10 (DS = 1.5), HAM 11 (DS = 1.6) and HAM 04 (DS = 2.6). The second dimension, SEC, calibrated with PEO standards.*

6.7. Conclusion

A novel, reliable and robust multi-dimensional LC method was developed for the separation of unmodified HA and HA modified with acrylate functionalities in the range of DS = 0 to 3.1. The developed method was able to separate HAM according to chemical composition followed by the separation based on molar mass. The multi-dimensional LC analyses of the polymers demonstrated the complexity of these natural polymers as the samples not only showed a distribution with regard to DS, but also a bimodal molar mass distribution within a specific DS. Even though the analysis and characterization of the HA polymers had been

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shown to be challenging due to HA being inherently heterogeneous, the new formulated approach proved to be able to elucidate the complex structure of HA, which is required for the understanding and establishment of structure-property relationships.

6.8. References

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Chapter 7

Summary, Conclusion and Future Work

In this chapter, a brief summary and conclusion of the results obtained during the study are presented along with recommendations for future work.

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7.1. Summary and Conclusion

The aim of the study was to develop advanced fractionation methods for the analysis of complex hyaluronic acid (HA) derivatives. Such derivatives were prepared by attaching acrylate groups to the hyaluronic acid backbone and exhibited distributions in chemical composition (degree of substitution) and molar mass.

The aim of the study was achieved by the application of advanced analytical techniques such as multi-dimensional liquid chromatography. The coupling of different liquid chromatography techniques, such as gradient HPLC and SEC, made the analysis of the inherently complex HA polymers possible. The HA polymers received from L'Oréal (Paris, France) were heterogeneous as the HA backbone was modified with acrylate moieties to various degrees. Comprehensive information with regards to two different distributions, chemical composition and molar mass, was acquired using multi-dimensional LC coupled with an ELSD. The information acquired with regards to the unmodified HA and modified HA could assist in the production of better consumer-related products.

The summarized results and conclusions of the study are as follow:

A suitable solvent system for HA and HA derivatives that was compatible with the HPLC experimental setup and the ELSD detector was obtained and the solubility behaviour of derivatives with different degrees of substitution (DS) was investigated. The solvent system most suited for the dissolution of HA, in both the modified and unmodified state, contained a volatile salt. It was found that the addition of a salt at low concentration enhanced the solubility of the HA polymers. The solubility of the polymer was dependent on the DS, as the polymer became more hydrophobic with an increase in DS. The solubility study showed that the solvent system was more favourable for the lower DS HA polymers. Determination of a solvent system that was suitable for dissolving the HA polymers, be compatible with the stationary phase and ELSD, was facilitated by dynamic light scattering (DLS) investigations. DLS allowed quantitative analysis of the behaviour of the polymer in solution as well as to determine if the formation of aggregates is influenced and possibly minimized. It was concluded that ACN:H_2O (40:60, vol. %) with a volatile salt was a suitable solvent system, even though the presence of aggregates was still observed to a low degree. DLS analysis

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showed very small differences between unfiltered and filtered samples, which effectively means that sample losses by filtration were low.

Advanced analytical LC techniques based on HPLC and SEC were successfully developed and efficiently used to characterize complex modified HA and unmodified HA according to chemical composition and molar mass distributions. SEC analyses were conducted with dRI and ELSD detection and the system was calibrated with narrowly dispersed poly(ethylene) oxide calibration standards. The effect of salt added to the mobile phase on the molar mass determination was investigated. Without salt, it was found that the HA polymers eluted outside the separation range of the SEC system. However, with the addition of a volatile salt to the sample solvent and the mobile phase, the HA polymers eluted within the exclusion limits of the SEC column set. It was concluded that the addition of salt assisted in reducing the formation of aggregates. Further investigations into the type of volatile salt and the concentration of the salt were conducted and it was found that the type of salt used and the concentration thereof, had no significant effect on the molar mass determined. From the solubility studies conducted in combination with the SEC results, it was concluded that ACN:H_2O (40:60, vol. %) with 0.02 M ammonium acetate was a suitable solvent and mobile phase system for both the unmodified and modified HA irrespective of DS.

HPLC investigations in the liquid adsorption (LAC) mode for the separation of the HA polymers according to chemical composition, were performed on a non-polar C8 column with solvent gradient elution. The developed gradient HPLC method with ACN/water as the mobile phase successfully separated the unmodified HA from the modified HA and the elution behaviour of the modified HA was according to DS. The gradient HPLC method was optimized using a stepwise gradient and baseline separation was achieved. The optimized method improved the resolution while the elution behaviour of the HA polymers was maintained. The low DS components eluted first, while the higher DS components eluted at a later elution volume. The HA samples were fractionated to investigate the chemical composition (DS) of each fraction. The higher DS HA polymers exhibited three different elution peaks. With the aid of offline ^{1}H-NMR the two late eluting peaks were assigned to lower DS components and higher DS components, respectively. The first eluting peak was

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concluded to be a combination of unmodified HA and modified HA (breakthrough effect) and should thus be investigated further.

The correlation between chemical composition and molar mass was achieved by the online coupling of the individual LC methods developed in a comprehensive 2D HPLC x SEC setup. The contour plot of the unmodified HA (HA 1) showed a broad molar mass distribution for the polymer. Samples HAM04, HAM09 and HAM10 were derived from HA 1. In the contour plots of these modified HA polymers, several components were observed which related to the DS distribution and molar mass distribution. In the majority of the modified HA polymers, bimodalities in molar mass were observed for components with different DS. This confirmed the complexity of the molecular structure of the acrylate-functionalized modified HA and the relevance of the analysis thereof. The second dimension (SEC) of the 2D-LC system was calibrated using poly(ethylene) oxide calibration standards. The molar masses obtained for the HA correlated well with the molar masses obtained from the one dimensional analyses.

7.2. Future Work

Suggested recommendations for future work include the following:

(a) Optimize the stepwise gradient method for further improvement of the separation achieved according to DS. A possible suggestion would be to also add the salt to the gradient ACN/Water mobile phase in RP-LC to investigate the effect it could have on the elution behaviour of the polymers.
(b) Optimize the 2D-LC method with regards to efficiency and time.
(c) Compare the two HPLC approaches, in other words, the separation achieved on a non-polar reversed stationary phase to the separation achieved using a polar normal stationary phase.
(d) Couple 2D-LC with FTIR in order to obtain quantitative compositional information with regards to the HA polymers.
(e) Determine the absolute molar masses of the unmodified and modified HA by SEC-MALLS using the current solvent system developed.

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(f) Determine the molar mass distribution of the unmodified and modified HA by Asymmetrical Field Flow Fractionation (AF^4)

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Appendix A

Table A.1: Solubility study at 25°C with a sample concentration of 1.0 mg.mL^{-1}.

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	25 ℃	25 ℃	25 ℃	25 ℃	25 ℃
1,4-Dioxane:H_2O (20:80)	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)	+	+/-	+	+	+
1,4-Dioxane:H_2O (50:50)	-	+/-	+/-	+/-	+
1,4-Dioxane:H_2O (60:40)	-	+/-	+/-	+/-	+/-
1,4-Dioxane:H_2O (80:20)	-	-	-	-	-
Acetonitrile:H_2O (50:50)	-	+	+	+/-	+

Table A.2: Solubility study at 40°C with a sample concentration of 1.0 mg.mL^{-1}.

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	40 ℃	40 ℃	40 ℃	40 ℃	40 ℃
1,4-Dioxane:H_2O (20:80)	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)	+	+	+	+	+
1,4-Dioxane:H_2O (50:50)	-	+	+	+/-	+
1,4-Dioxane:H_2O (60:40)	-	+	+	+-	+
1,4-Dioxane:H_2O (80:20)	-	-	-	-	-
Acetonitrile:H_2O (50:50)	-	+	+	+/-	+

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Table A.3: Solubility study at 25°C with a sample concentration of 1.0 mg.mL^{-1}(0.1 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	25 °C	25 °C	25 °C	25 °C	25 °C
1,4-Dioxane:H_2O (40:60)/ 0.1 M ammonium formate	+	+/-	+	+/-	+/-
1,4-Dioxane:H_2O (40:60)/ 0.1 M ammonium chloride	+	-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.1 M ammonium trifluoro acetate	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.1 M ammonium acetate	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.1 M formic acid	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.1 M trifluoroacetic acid	+	+/-	+	+	+

Table A.4: Solubility study at 40°C with a sample concentration of 1.0 mg.mL^{-1} (0.1 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	40 °C	40 °C	40 °C	40 °C	40 °C
1,4-Dioxane:H_2O (40:60)/ 0.1 M ammonium formate	+	+/-	+	+	+/-
1,4-Dioxane:H_2O (40:60)/ 0.1 M ammonium chloride	+	-	+	+/-	+
1,4-Dioxane:H_2O (40:60)/0.1 M ammonium trifluoroacetate	+	+/-	+	+/-	+/-
1,4-Dioxane:H_2O (40:60)/0.1 M ammonium acetate	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.1 M formic acid	+	+/-	+/-	+/-	+/-
1,4-Dioxane:H_2O (40:60)/0.1 M trifluoroacetic acid	+	+/-	+/-	+/-	+/-

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Table A.5: Solubility study at 40°C with a sample concentration of 1.0 mg.mL^{-1} (0.02 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	40 ℃	**40** ℃	**40** ℃	**40** ℃	**40** ℃
1,4-Dioxane:H_2O (40:60)	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.02 M ammonium acetate	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.02 M ammonium formate	+	+/-	+	+	+/-
1,4-Dioxane:H_2O (40:60/0.02 M ammonium trifluoroacetate	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.02 M formic acid	+	+/-	+	+	+
1,4-Dioxane:H_2O (40:60)/0.02 M trifluoroacetic acid	+	+/-	+	+	+

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Table A.6: Solubility study at 25°C with a sample concentration of 1.0 mg.mL^{-1} (0.1 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	25 °C	25 °C	25 °C	25 °C	25 °C
Acetonitrile:H_2O (50:50)/0.1 M ammonium formate	-	+/-	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium chloride	-	+/-	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium trifluoroacetate	-	+/-	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium acetate	-	-	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M formic acid	-	+/-	+	+	+
1,4-Dioxane:H_2O (50:50)/0.1 M trifluoroacetic acid	-	+/-	+	+	+

Table A.7: Solubility study at 40°C with a sample concentration of 1.0 mg.mL^{-1} (0.1 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	40 °C	40 °C	40 °C	40 °C	40 °C
Acetonitrile:H_2O (50:50)/0.1 M ammonium formate	+	+	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium chloride	+	+	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium trifluoroacetate	-	+	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M ammonium acetate	-	-	+	+	+
Acetonitrile:H_2O (50:50)/0.1 M formic acid	-	+/-	+	+/-	+/-
1,4-Dioxane:H_2O (50:50)/0.1 M trifluoroacetic acid	-	+/-	+	+/-	+/-

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Table A.8: Solubility study at 40°C with a sample concentration of 1.0 mg.mL^{-1} (0.02 M salt)

Solvent	HA 1	HAM 01 (DS: 3.1)	HAM 06 (DS: 0.8)	HAM 09 (DS: 2.5)	HAM 10 (DS: 1.5)
	40 ℃	**40** ℃	**40** ℃	**40** ℃	**40** ℃
Acetonitrile:H_2O (40:60)	-	+	+	+	+
Acetonitrile:H_2O (40:60)/0.02 M ammonium acetate	+	+/-	+	+	+
Acetonitrile:H_2O (40:60)/0.02 M ammonium trifluoroacetate	+	+/-	+	+	+
Acetonitrile:H_2O (40:60)/0.02 M ammonium formate	-	-	+/-	+/-	+
Acetonitrile:H_2O (40:60)/0.02 M formic acid	-	+/-	+	+	+
Acetonitrile:H_2O (40:60)/0.02 M trifluoroacetic acid	-	+/-	+	+	+

+ soluble

+/- partially soluble (opaque)

- insoluble